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13. ABSTRACT *(Maximum 200 Words)*

Our research is focused on the development of a PSA directed immunotherapy for prostate cancer. Through repetition of the experiments with additional controls, we confirmed our conclusion of last year that PSA can serve as a tumor rejection marker in the PSA transgenic mouse in spite of the potential tolerance to the PSA protein due to its self-status. Vaccination with a PSA expressing human tumor cell line elicited a specific anti-PSA response that protected mice from a challenge of PSA/OM-2 but not the control vector/OM-2 cell line. We identified PSA peptides that are immunogenic in the human HLA-A*0201 haplotype. Of nine candidate peptides five have been proven to be immunogenic in the HLA-A2Dd transgenic mouse after vaccination with peptide pulsed dendritic cells. These five peptides were further characterized with binding assays to reveal that four were exclusively bound by the A2 MHC molecule and one by the murine H-2D^b MHC molecule. Individually, all five peptides were shown to be efficacious in inducing an anti-PSA immune response capable of protecting 33-60% of A2Dd transgenic mice challenged with the tumor cell line, PSA/EL4A2Kb. The results of these experiments allow us to continue to pursue an anti-PSA directed prostate cancer immunotherapy

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Introduction

Prostate cancer is a relatively common disease of men that carries a lifetime risk of about ten percent and is reported to be present in eighty percent of eighty years olds at autopsy. Although the majority of the individuals who contract the disease are not affected by it, the widespread occurrence of the cancer enables it to account for about forty thousand deaths a year in the United States of America alone. Surgical resection can cure early disease but is often avoided due to the usual slow growth of the tumors and complications of the operation including impotence and incontinence. In the absence of a curative treatment for metastatic disease, a potent immunotherapy that would eradicate the prostate cancer cells would greatly benefit those individuals suffering from the malignancy. We have initiated experiments to develop a vaccine to elicit an anti-prostate cancer immunotherapy capable of destroying existing tumor cells that express prostate specific antigen (PSA). Our research has focused on both demonstrating the possibility of inducing such an immunity in a situation with potential tolerance to the antigen and creating an immunotherapy restricted to the human HLA-A*0201 haplotype capable of eliminating pre-existing tumors. Should we be successful, the techniques of our vaccine could be applied to all the human haplotypes upon the determination of the immunogenic peptides relevant for each haplotype. More importantly, the results of our experiments may lead directly to phase I clinical trials for eventual evaluation of the vaccine in treating human prostate cancer.

Body

The following report represents the work completed to date that essentially completes the first two technical objectives and part of the third. The original grant was divided into three technical objectives designed to initiate the development of a prostate cancer immunotherapy targeting the human prostate specific antigen protein found in most prostatic adenocarcinomas. The first objective strived to prove the possibility of targeting the PSA protein of tumors in mice who express PSA in their prostates due to germline inclusion of the PSA gene fused to the prostate specific probasin promoter. The second objective was designed to identify epitopes of the PSA protein that were restricted to the human HLA-A*0201 haplotype and immunogenic in the HLA-A*0201/Dd transgenic mouse. Finally, the third objective would evaluate the immunogenic peptides for their efficacy in eradicating PSA expressing tumors with the ultimate goal of creating an optimized vaccine that would eliminate preexisting tumors in hybrid mice containing both the PSA and HLA-A*0201/Dd transgenes. The successful completion of these experiments was originally argued to establish the tools necessary to translate this work into phase I clinical trials.

For technical objective one, this report includes the data from repeated experiments that confirm the results initially reported in the first annual report where we demonstrated the ability to induce a protective anti-PSA effect. In addition, this report also details the experiments that essentially completes the second technical objective where four PSA peptides were found to be immunogenic in the HLA*A0201/Dd transgenic mouse. Finally, we report on the experiments performed under technical objective three where we show the ability of the four identified PSA peptides plus one additional peptide with great similarity to one of the original four to elicit a protective anti-PSA immunity in A2Dd transgenic mice challenged with PSA expressing tumor cells.

Unfortunately, our progress has been delayed due to the outbreak of murine hepatitis virus (MHV) at Loyola University Chicago. Although this virus has not confounded the data generated to date, it needs to be eradicated from the animal colonies. This entails either a 'burn-out' period where breeding mice are housed for twelve weeks until the virus is no longer infectious or the rederivation of the colony using fetal transplant of transgenic mouse embryos into pseudopregnant, clean mice. Both strategies are currently employed to insure the most rapid return of uninfected mice to the laboratory for the prompt resumption of the experiments. The A2Dd mice may also be obtained in the near future from a vendor (Jackson Laboratories) due to their recent inclusion of the mouse strain in their inventory.

Technical Objective One

Technical objective one was divided into three specific tasks to determine the feasibility of targeting the PSA protein in a setting where it is considered a self-protein. The tasks involved task 1, the creation of the model, task 2, the vaccination of PSA transgenic mice with PSA DNA or emulsified PSA protein with subsequent challenge with syngeneic PSA expressing tumor cells and task 3, the measurement of the efficacy of any induced immunity. The first annual report demonstrated the completion of technical objective one with unsuccessful results that prompted a change in the experimental protocol. We switched from the vaccination strategies of DNA or protein

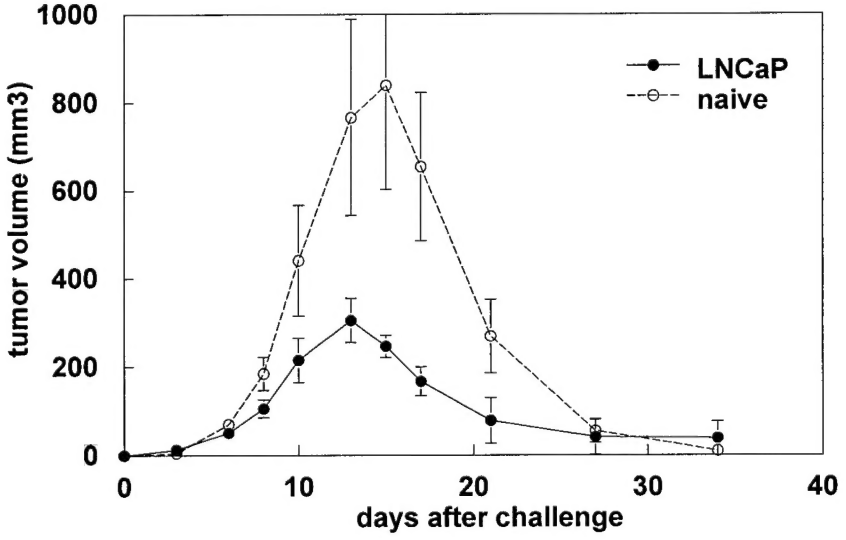
vaccination to the subcutaneous injection of human prostate cancer cells, LNCaP, and were able to show a protective effect in PSA transgenic mice challenged with PSA expressing tumor cells, PSA/OM-2 versus mice challenged with the parental, PSA negative tumor cells. Since reporting this experiment in the first annual report, we have since repeated the experiment with additional controls and found similar conclusions that are detailed below.

The experiment originally reported in the first annual report involves the use of the human prostate cancer cell line, LNCaP, which has been shown to highly express PSA. PSA transgenic mice were vaccinated with ten million LNCaP cells four times separated by three weeks. After the final vaccination, mice were challenged with either PSA/OM-2 or the parental cell line, OM-2, and followed for tumor progression. The original results showed that the mice challenged with the OM-2 cell line grew much larger tumors than those mice challenged with PSA/OM-2. We concluded from these experiments that the vaccination of the LNCaP tumor cell line induced a protective anti-PSA response that did not affect the parental cell line.

Buoyed by the initial results of the LNCaP experiments, we included two new controls to insure the accuracy of our conclusions. First, the parental cell line, OM-2, used as the control lacked the vector-associated proteins from the plasmid used to construct the PSA/OM-2 cell line. Thus to avoid confounding results via a tumor rejection immunity against the vector associated protein, the parental cell line was transfected with the empty vector to create the ideal control cell that differed only through the absence of PSA expression.

The second control added involved the inclusion of mice not vaccinated with LNCaP cells but challenged with either the PSA/OM-2 or Vector/OM-2 cells. This control was included to insure that the immunization with the LNCaP cell line did not induce a non-specific anti-neoplasm immunity through increasing the level of the immune system's responsiveness. Thus comparison of tumor progression in PSA transgenic mice should only show protection in mice vaccinated with LNCaP cells and challenged with PSA/OM-2.

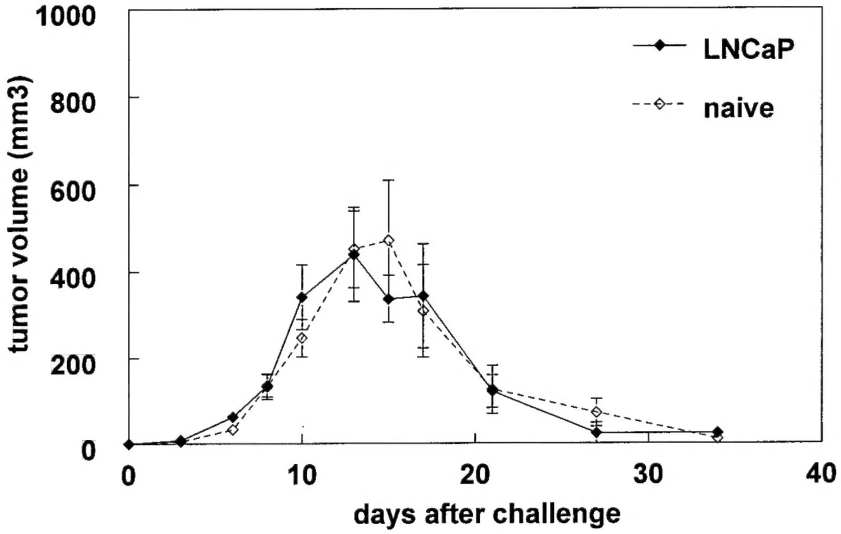
Figure 1. Challenge of naive or LNCaP vaccinated PSA transgenic mice with PSA/OM-2 showing decreased tumor size in vaccinated mice.



As predicted, the inclusion of these two controls did not alter our conclusions from the two sets of experiments that repeated the initial experiment. In both cases, a protective effect

was only found in mice vaccinated with LNCaP cells and challenged with PSA/OM-2 (Figure 1). The included controls further strengthened our argument since the vaccination of mice with LNCaP cells did not alter the growth characteristics of the vector/OM-2 cells when compared to non-vaccinated mice (Figure 2). Unfortunately none of our experiments were able to show the presence of specific CD8 positive T cells using either ^{51}Cr release or ELISPOT assays which precluded us from defining the mechanism of this anti-PSA response. In lieu of this, we are able to conclude that vaccination of PSA transgenic mice with the LNCaP cells did induce an anti-PSA response despite the self-antigen status of the PSA transgene.

Figure 2. Challenge of naive vs LNCaP vaccinated PSA transgenic mice with vector/OM-2 showing similar tumor growth curves.



Due to the regressor nature of the PSA/OM-2 cell line, we additionally set forth to develop a progressor cell line that expressed PSA and was tumorigenic in the PSA transgenic mouse. We reported in the first annual report the successful creation of just such a cell line, PSA/OM-95 2A. Although this cell line was found to express the RNA for PSA via RT-PCR, we were astonished to find that repeated attempts to detect the expressed PSA protein via a sensitive sandwich ELISA or western blots failed. Thus inexplicably this cell line was found not to be useful for our purposes. We plan on retransfecting the original progressor parental strain again to establish the progressor, PSA expressing tumor cell line syngeneic in the PSA transgenic mouse line.

Technical Objective Two

Technical objective two was also divided into three tasks designed to identify the HLA-A*0201 (A2) restricted epitopes of the PSA protein that are immunogenic in the HLA-A*0201/D^d (A2Dd) transgenic mouse. Task one was designed to create plasmids containing minigenes encoding one of nine candidate peptides predicted through competition assays to bind the A2 MHC molecule (see Table 1). Task 2 involved the vaccination of the A2Dd transgenic mouse with either the minigene plasmids or peptides emulsified in incomplete Freund's adjuvant (IFA). Task three would complete this technical objective through the measurement of the induced immune response using ^{51}Cr release assays thus identifying which of the nine candidate peptides were also immunogenic in the A2Dd transgenic mouse.

Table 1. Competition data and sequences for the peptides used in this study (PSA-7 not analyzed).

Peptide	Sequence	IC50 (nM)
PSA-1	KLQCVDLHV	79
PSA-2	MLLRLSEPA	132
PSA-3	FLTPKKLQCV	161
PSA-4	VLVHPQWVL	417
PSA-5	VTWIGAAPL	455
PSA-6	KLQCVDLHVI	649
PSA-7	VISNDVCAQV	N.D.
PSA-8	FMLCAGRWT	725
PSA-9	VVFLTLSVTWI	847

The first annual report recounted the need to change our originally stated approach to a vaccination strategy that used the infusion of peptide pulsed dendritic cells (DC). We did not create the peptide minigenes due to the lack of results seen with the initial DNA vaccinations in the PSA transgenic mouse and the growing support of a DC based approach in the literature. The use of the s.c. injected emulsified peptides was also attempted in the laboratory but also failed to produce results and was subsequently discontinued. Using the DC based approach, the first annual report described the identification of peptides PSA-2 and PSA-7 as immunogenic in the A2Dd transgenic mouse. Contained below are the results obtained since the first annual report showing the further characterization of the nine candidate peptides and the immunogenicity of two additional peptides.

To further characterize the nine candidate peptides originally selected for their low IC_{50} values in competition assays, we employed two binding assays to establish the extent each peptide binds to either the human A2 MHC or murine MHC molecules. The human assay utilizes the human, TAP deficient cell line, T2, that cannot endogenously load their MHC molecules creating surface MHC molecules with short half-lives. Exogenous loading of these molecules with added peptide stabilizes these molecules and allows for the increased concentration on the cell surface that allows for quantification via flow cytometry. The level of surface MHC molecules directly correlates with the affinity of the added peptide. The results of our experiments with at least three repetitions per peptide reveals that peptides PSA-1, PSA-3, PSA-6 and PSA-7 strongly bound the A2 MHC molecule as shown in figure 3.

Figure 3. T2 Binding data for all nine PSA peptides All data normalized to F.I. of HPV 16 E7 86-93

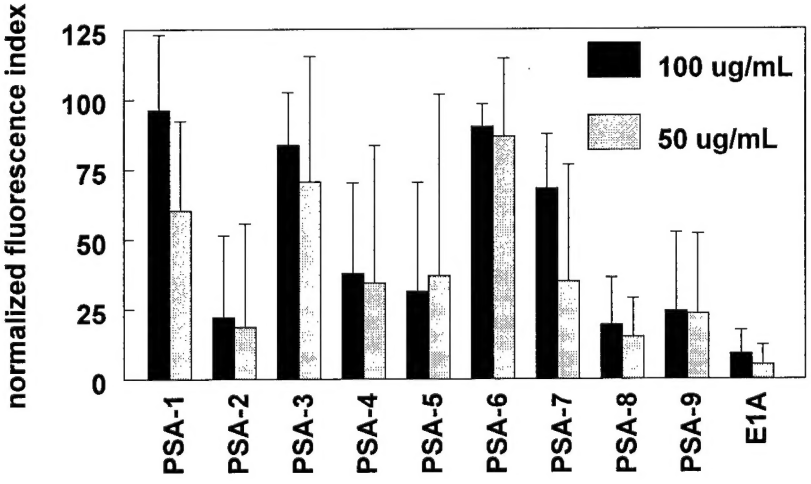


Figure 5. RMA-S binding assay for H-2K^b data normalized to FI of the sendai virus S9 peptide

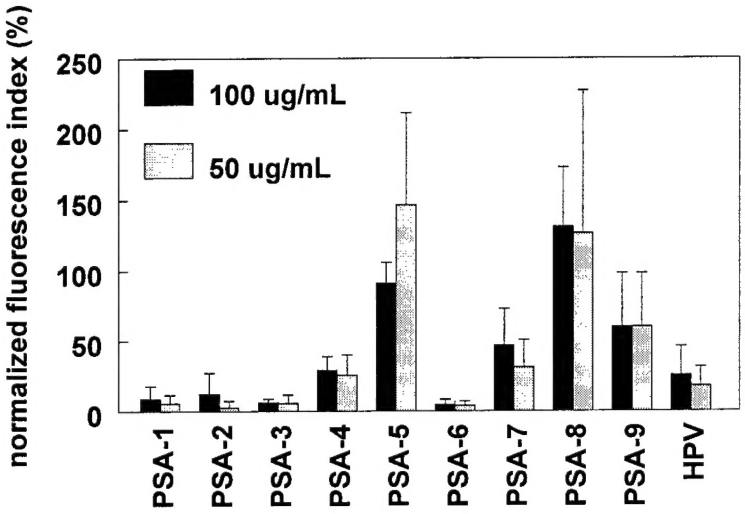
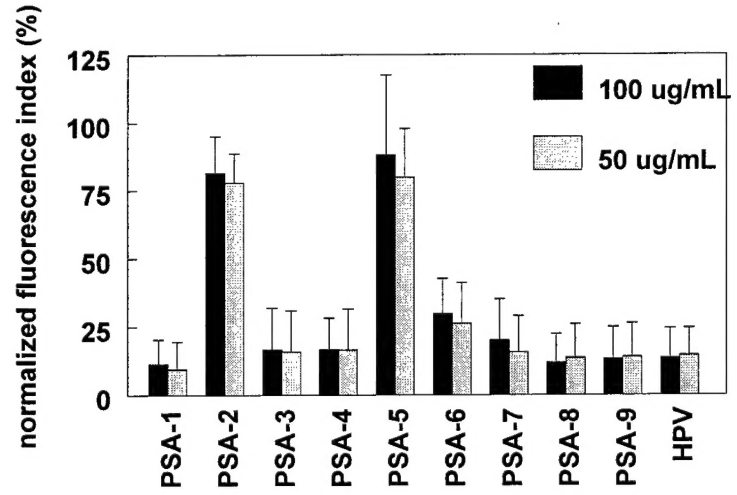


Figure 4. RMA-s binding assay for H-2D^b data normalized to F.I. of Ad5 E1A peptide

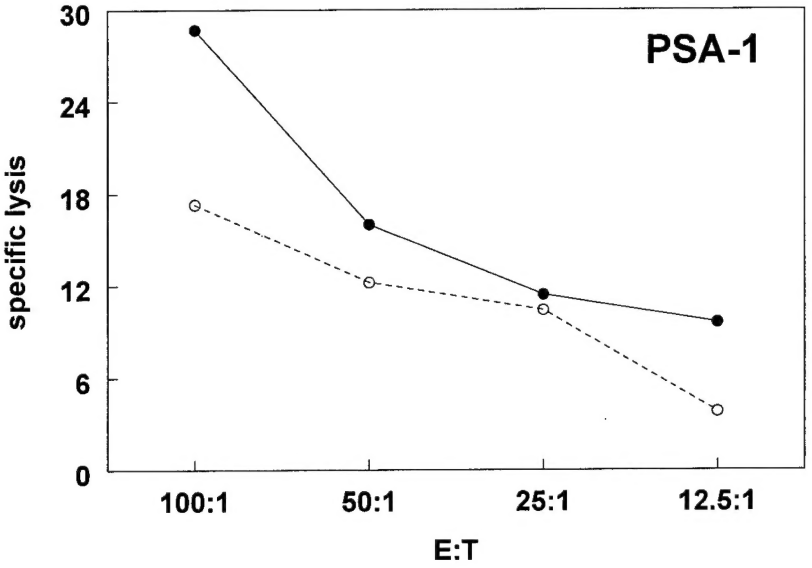


Additionally, we investigated the ability of each peptide to bind the murine MHC molecules in the murine binding assay to establish the range of binding affinities for all MHC molecules present in the A2Dd mouse model. These experiments were conducted in the same manner as the T2 binding assay but with the mouse cell equivalent, RMA-S. Herein we discovered the ability of PSA-2 and PSA-5 to bind the murine H-2D^b molecule and the ability of PSA-5 and PSA-8 to bind the murine H-2K^b MHC molecule as seen in figures 4 and 5.

The conclusions from these experiments revealed PSA-1, PSA-3, PSA-6 and PSA-7 as candidate A2 binding peptides and PSA-2, PSA-5 and PSA-8 as candidate murine MHC binding peptides. The discrepancy between the results from the competition assay and our binding assays was hypothesized to be explained by different batches of peptide used by the company who performed the competition assay. We attempted to test this through the parallel testing of two different sources of peptide and found that non-HPLC purified peptides gave similar results when compared to HPLC purified peptides. In light of the above binding data and immunogenicity data found below, we chose to continue our experiments with PSA-1, PSA-2, PSA-3 PSA-6 and PSA-7 peptides.

As described in the first annual report, task 2 and 3 were partially completed with the demonstration of the immunogenicity of peptides PSA-2 and PSA-7. Further testing with the same experimental design revealed the ability of PSA-1 and PSA-3 to induce a

Figure 6. ^{51}Cr release data for PSA-1. Open symbols represent control target cells whereas closed represent peptide pulsed target cells.



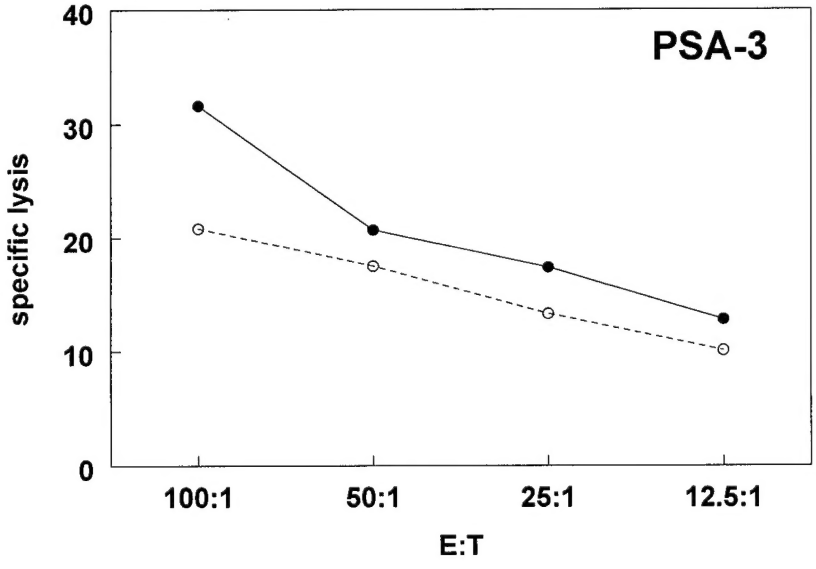
specific CD8 positive immune response (see Figures 6 and 7). Briefly, these experiments involved the vaccination of all mice with the helper epitope from the human hepatitis B virus core antigen (HBVcAg) emulsified in IFA ten days prior to vaccination with the DCs. The mice received two immunizations of one half million DCs pulsed with both the HBVcAg helper peptide and relevant PSA peptide with the injections separated by two weeks. Seven to fourteen days later, splenocytes were removed from the mice, restimulated in vitro with peptide loaded syngeneic splenocytes and used in a standard ^{51}Cr release assay.

From the original nine peptides, we selected PSA-1, PSA-2, PSA-3 and PSA-7 for future use in our experiments based on their binding affinities and immunogenicity. Due to the binding affinity of PSA-6 that is stronger than PSA-1 and the fact that its sequence is PSA-1 plus one amino acid, we chose to include it also in our future experiments despite the lack of immunogenicity data. The selection of these five peptides completes technical objective two and allowed us to continue our work in technical objective three.

Technical Objective Three

Technical objective three was initially separated into five tasks intended to combine the results of the first two technical objectives for the eventual creation of an anti prostate cancer immunotherapy that targets PSA. The initial task called for the creation of two PSA expressing tumor cell lines, one for use in the A2Dd transgenic mouse and one for use in the F1

Figure 7. ^{51}Cr release data for PSA-3. Open symbols represent control target cells whereas closed symbols represent peptide pulsed target cells.



hybrids of a cross of the PSA and A2Dd transgenic mice. Additionally the first task was established to determine the optimal vaccine from the results of the first two technical objectives. The second task involved the actual vaccination of the mice with the vaccination strategy determined from above with the subsequent measurement of the efficacy of the induced immunity through tumor protection studies. Tasks 3, 4 and 5 were meant to evaluate the memory response, therapeutic ability and chemotherapeutic augmentation of any therapeutic ability, respectively. Work from the first annual report had only satisfied the requirement to produce the tumor cell line for use in the A2Dd transgenic mouse. Since that time, we have been able to fulfill parts of this technical objective as described below with minor adjustments until the outbreak of MHV halted our ability to continue.

Due to the changes from technical objective two where we had to alter our vaccination approach to a DC based vaccine, the "optimal vaccine" was not able to be determined. It was determined to be more important to assess each peptide independently and then combine the relevant epitopes in an optimal vaccine rather than assume the potency of each and include them without the supporting data. Thus task two was altered to reflect this new strategy with the assumption that after completion of these studies, the optimal vaccine would be subsequently tested with the inclusion of all peptides shown to induce a protective anti-PSA immunity in tumor challenge experiments.

As will be discussed below, we were successful in evaluating the efficacy of the five selected peptides in inducing a protective anti-PSA immunity thus identifying them all as candidates for inclusion in the optimal vaccine. Unfortunately, we were forced to stop prematurely due to the MHV outbreak that prevented us from evaluating the potency of the optimal vaccine. Additionally, the outbreak required us to destroy our production of the tumor cell line for use in the PSA/A2Dd F1 hybrid mice due to potential viral contamination (discussed below). We chose to focus our efforts on the A2Dd mouse instead of attempt these experiments in the F1 hybrids of the PSA and A2Dd transgenic mice due to the potential interference the self-status of the PSA protein may have on the experiments. With the successful determination of the optimal vaccine, these experiments will be translated into the PSA/A2Dd transgenic model to test the vaccine's efficacy in lieu of the potential tolerance to PSA in the double transgenic mouse. Finally, since tasks 3, 4 and 5 involve the use of the optimal vaccine strategy, the absence of its exact specifications has precluded our ability to complete these tasks at this time.

Before the MHV outbreak, we were able to test the efficacy of each peptide using our vaccination strategy in tumor protection experiments. These experiments used the A2Dd transgenic mouse model with the syngeneic, PSA expressing tumor cell line described in the first annual report, PSA/EL4A2Kb. The experimental design included the vaccination of all mice with the HBVcAg helper peptide to sensitize the mouse's T helper cells for their eventual role in maturing the subsequently injected DCs pulsed with both the HBVcAg helper and PSA peptides. Ten days later, the mice received two injections of five hundred thousand DCs pulsed with both the HBVcAg and PSA peptides with the injections separated by two weeks. Control mice received the exact same vaccine except for the substitution of the immunogenic HPV 16 E7 86-93 peptide for the PSA peptide. Fourteen days after the final vaccination, all mice were challenged with the empirically determined dose of PSA/EL4A2Kb tumor cells known to induce tumors in

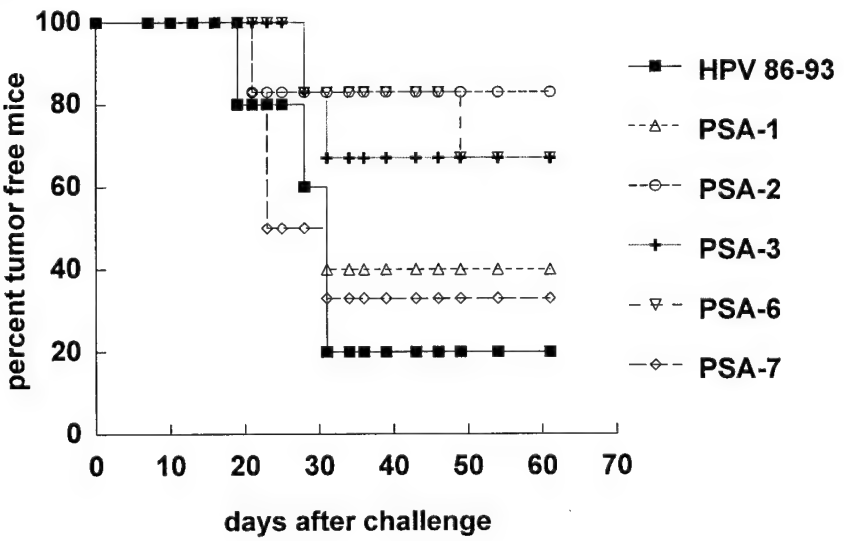
100% of challenged mice. Mice were followed for time to tumor development and tumor size.

Table 2. Cumulative data of the five experiments performed to assess the ability of the peptides to induce a protective anti-PSA immunity capable of preventing the outgrowth of PSA/EL4A2Kb tumors to 60 days. Each block contains number of tumor free mice at day 60 over number of mice in each group with percent tumor free at sixty days beneath.

Peptide	EXP. #1	EXP. #2	EXP. #3	EXP. #4	EXP. #5		TOTALS
PSA-1	N/A	3/4 75%	N/A	2/5 40%	2/6 33%		7/15 47%
PSA-2	2/5 40%	0/4 0%	N/A	5/6 83%	0/5 0%		7/20 35%
PSA-3	N/A	N/A	1/4 25%	4/6 67%	4/5 80%		9/15 60%
PSA-6	N/A	N/A	N/A	4/6 67%	1/5 20%		5/11 54%
PSA-7	1/5 20%	N/A	2/4 50%	2/6 33%	2/5 40%		7/20 35%
HPV 86-93	0/4 0%	0/4 0%	0/4 0%	1/5 20%	1/5 20%		2/22 9%

The cumulative results of these experiments can be found in table 2 with a characteristic Kaplan-Meyer survival curve found in figure 8. These results show that all five peptides induced a protective anti-PSA immunity when compared to the controls. From these results we were able to establish the rank order potency of the peptides in preventing the outgrowth of the PSA expressing tumors as PSA-3>PSA-6>PSA-1>PSA-2=PSA-7. We believe that the immunity induced from vaccination with PSA-1 or PSA-6 probably induces a similar set of T cells and that the small difference in efficacy may be due to the increased affinity of PSA-6 for the A2 molecule as seen in our binding studies. In addition, we believe that the immunity induced by PSA-1, PSA-3, PSA-6 and PSA-7 are through the chimeric A2Dd

Figure 8. Kaplan-Meyer survival analysis of A2Dd transgenic mice vaccinated with peptide peulsed DCs and challenged with PSA/EL4A2Kb



MHC molecule whereas the immunity induced by PSA-2 is through the H-2D^b molecule based on our previous T2 and RMA-s binding assays.

In summary, the results obtained in technical objective three to date have established the identity of four A2 restricted and one H-2D^b restricted peptides for inclusion in future experiments. The lack of complete protection from any of the peptides indicates the need to optimize the vaccine strategy. We decided on two methods to attempt to increase the potency of the vaccination strategy but were prevented from evaluating the value due to the MHV outbreak. The first strategy involved the combination of the five peptides into one DC based vaccine to test the cumulative effects of all peptides. The second approach involved the creation of the herein previously maligned DNA minigene strategy with specific modifications to enhance its usefulness.

The combination of each peptide into one DC based vaccine may increase the efficacy to provide the desired 100 % protection from challenge with PSA expressing tumor cells. This approach may enhance the potency via increasing the number of reactive T cells since each peptide may elicit a distinct set of T cells. Furthermore, the increased diversity of the response may also prevent the tumor escape through the point mutation of the epitope targeted through a single peptide vaccination approach.

The second approach to attempt the optimization of the vaccination strategy involves the creation of a minigene plasmid encoding the sequence of each epitope. Although previously proven to fail in our hands, parallel work in the lab introduced important modifications that enhanced the efficacy of the approach. These modifications entailed the necessity for spacers between the epitopes and the addition of a ubiquitination signal. This method has now been shown to effectively eradicate one week old tumors in a human papilloma virus model where the target is the E6/E7 viral protein. Using this approach then may also prove to be equally efficacious in the PSA model.

With the optimization of the vaccine strategy, tasks 3, 4 and 5 as originally proposed will then be undertaken for the completion of this technical objective and ultimately the completion of the grant proposal. The unfortunate set back involving the MHV infection has prevented us from performing the final experiments on this project involving mice since every experiment requires transgenic mice. The eventual return of clean mice via burnout/rederivation or forthcoming ability to purchase these transgenic mice through specific vendors will allow the continuation of these final experiments.

At this time, two other points from the original grant need comment due to specific alterations from the originally stated tasks including the use of F1 hybrids of the PSA and A2Dd transgenic mice and development of a tumor cell line for use within these F1 hybrids. Due to the potential for interference in the development of an anti-PSA immune response to tolerance if PSA is a self-antigen, we decided to initially conduct our experiments in mice without the germline inclusion of the PSA gene. Once the optimal vaccine is established in a non-PSA expressing A2Dd transgenic mouse, we feel it would be logical to apply the vaccine in the PSA/A2Dd transgenic F1 hybrids. This will allow us to evaluate any barriers the potential tolerance to PSA may have on the efficacy of the vaccine rather than prevent the discovery of the relevant epitopes for inclusion. Although we originally stated in the grant that these experiments would occur in parallel, we feel this plan will afford us a better opportunity to accurately analyze each peptide's efficacy.

The second point that we specifically altered from our original grant proposal involves the creation of a tumor cell line for use in the F1 hybrid mice from the cross of the PSA and A2Dd transgenic mice. Since both mice are derived from mouse strains with different haplotypes, we need to create a tumor cell line that will express both alleles of the F1 hybrids. In order to create this syngeneic line, we had originally planned on transforming primary prostate cells isolated from a PSA/A2 F1 hybrid mouse with oncogenes or viral proteins. In light of poorly established methodology to complete this task with primary prostate cells and the attainment of the TRAMP mouse line, we chose to alter our approach to an established method. Through a collaboration with Dr. Eugene Kwon M.D. we obtained the TRAMP mouse line that spontaneously develops prostate cancer through the germline inclusion of the SV40 large and small T genes on the prostate specific probasin promoter. The successful breeding of the mice to obtain a triple transgenic will produce the desired tumor cell line when it spontaneously develops prostate cancer that concurrently expresses both the PSA and A2Dd transgenes. We had crossed the PSA/A2 F1 hybrid mice with homozygous TRAMP mice when the MHV outbreak forced us to destroy the litters. Once we obtain clean mice, these experiments will proceed with the goal of creating a prostate tumor cell line that expresses both the chimeric A2Dd and PSA genes and is syngeneic in the F1 hybrids.

The information contained in the body of this annual report summarizes the progress made on the original proposal to date. We have successfully completed the first two technical objectives with adjustments made when the science demanded. Work on the third technical objective, although temporarily hampered due to the MHV outbreak, produced initial positive results allowing us to continue to pursue the originally stated goal of developing an anti-prostate cancer immunotherapy specifically targeting the PSA protein.

Key Research Accomplishments

1) Identification of two additional PSA peptides as immunogenic in the HLA-A* 0201/D^d transgenic mouse model.

2) Induction of protection against tumor growth through vaccination with various HLA-A* 0201 restricted PSA derived peptides in the previously established PSA/EL4A2 K^b tumor model.

Reportable Outcomes

Manuscripts, abstracts, presentations and grants:

- Two year Fellowship (07/01/00 – 06/30/02) from the American Foundation for Urologic Disease and American Urological Association. Title of the project: Cancer Prevention and Immunotherapy in a Chronic Prostate Cancer Model.

- Holt GE, Velders MP, Rudolf MP, Small LA, Provenzano M, Weijzen S, Da Silva DM, Visser M, Ter Horst SAJ, Brandt RMP and Kast WM: Critical dependence of the peptide delivery method on the efficacy of epitope focused immunotherapy. In: Peptide based cancer vaccines. Kast WM (ed) Eurekah.com, Austin, TX, USA. 56-72, 2000.

- Yang D, Holt GE, Rudolf MP, Velders MP, Brandt RMP, Kwon ED and Kast WM: Peptide vaccines. In: New vaccine technologies. Ellis RW (ed) Eurekah.com, Austin, TX, USA. 214-226, 2000.

Patents and licenses applied for and/or issued:

None

Degrees obtained that are supported by this award:

None

Development of cell lines, tissue, or serum repositories:

None

Informatics such as databases and animal models, etc:

None

Funding applied for based on work supported by this award

Applied for a DOD idea grant (PC 001063) entitled: PSA-specific and non-antigen specific immunotherapy in a clinically relevant chronic prostate cancer model (Score 2.5, not fundable).

Employment or research opportunities applied for and/or received on experiences/training supported by this award:

None

Conclusion

The goal of this project was to develop an immunotherapy for prostate cancer that would specifically target the expressed PSA protein of the tumor cells. Using both a PSA transgenic model to simulate the human condition where PSA is considered a self-antigen and the A2Dd transgenic mouse model to simulate the human immune system, we experimented with different vaccination strategies to establish both the feasibility of such an approach and the initial components of a potential vaccine. To test the feasibility of targeting the self-antigen, PSA, we had originally reported our preliminary success in inducing an anti-PSA response in the PSA transgenic mouse. In order to insure the accuracy of these results, this report demonstrated the repetition of the first experiment with additional controls yielding similar conclusions. Vaccination of PSA transgenic mice with the PSA expressing human prostate cancer cell line, LNCaP, protected them from subsequent challenges with the PSA-OM-2 cell line but not the control vector/OM-2 cell line. These results effectively complete technical objective one and provide evidence for the ability to raise a protective anti-PSA immunity in a setting where potential tolerance to PSA exists.

The other two technical objectives were designed to identify and test for the ability of A2 restricted PSA peptides to protect mice from a challenge with PSA expressing syngeneic tumor cells and ultimately treat pre-existing tumors. We were successful in increasing the number of known immunogenic peptides to four via ^{51}Cr release assays and show that those four peptides plus one more that closely mimicked one of the original four were able to elicit the protective anti-PSA response in the A2Dd transgenic mouse. Additionally, these peptides were further characterized by binding assays and four were found to exclusively bind the A2 MHC molecule whereas one peptide exclusively bound one of the murine MHC molecules, H-2D^{b}. Armed with these results, we were poised to initiate experiments to optimize the vaccination strategy and begin work in the F1 hybrids of a PSA and A2Dd transgenic mouse cross when MHV infected the colony.

This problem is currently being solved by rederivation and obtaining mice from commercial vendors. Current plans include the combination of the peptides into an optimized DC based approach or incorporation of minigenes encoding each peptide into a string bead approach for DNA vaccination. Successful optimization of the vaccine will allow us to test the strategy in a therapeutic setting and in the F1 hybrids of the PSA and A2Dd transgenic mouse cross. Successful completion of these experiments should provide the data necessary to translate this data into a phase I clinical trial for the treatment of prostate cancer.

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CHAPTER 4

Critical Dependence of the Peptide Delivery Method on the Efficacy of Epitope Focused Immunotherapy

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Introduction

Tumor immunotherapy describes the use of the immune system as a tool to eliminate cancer from the stricken patient. The theory contends that immunization against certain proteins either associated with or specific for the tumor will create a potent cytotoxic T lymphocyte (CTL) immunity able to selectively kill the cancer cells. The anti-tumor specificity of the T cells is retained in the ability of the CTLs T cell receptor (TCR) to recognize an eight to twelve amino acid peptide bound to the MHC class I molecules of the tumor cells. Epitope focused immunotherapy (EFIT) is an offshoot of tumor immunotherapy that strives to identify the sequence of protein derived antigenic peptides bound to the MHC molecules and then focus the immunization against these epitopes.

This Chapter deals with the many different epitope based immunization strategies and describes the characteristics of each that contributes or detracts from the overall success of the method. It has been organized into four sections each dealing with an alternative immunization strategy for epitope based vaccines including peptide vaccination, dendritic cell vaccination, DNA vaccination and recombinant virus vaccination. The section on peptide vaccination describes the use of synthetic peptides injected primarily subcutaneously in association with noncellular adjuvants. Dendritic cell (DC) vaccination will detail the ex vivo loading of DCs with antigen using a variety of methods for their eventual reinfusion. The injection of genetic constructs containing "minigenes" encoding each peptide makes up the third part concerning DNA vaccination. Finally, the use of viruses engineered to express the peptides comprises the last section entitled recombinant virus vaccination.

Regardless of the method of vaccination employed, the eventual goal of the research performed in this field is to produce a therapeutic vaccine able to selectively destroy the tumor cells with limited side effects. This vaccine should have a rapid onset of CTL induction to prevent the initiation or exacerbation of tumor related complications and check tumor growth so the kinetics of the tumor cell proliferation does not overwhelm the immune system as per certain viruses.[1] The composition of the vaccine may contain peptides restricted to the patient's MHC haplotypes from every tumor antigen of the targeted cancer arranged consecutively in a

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"beads on a string" construct. The combination of all possible epitopes likely expressed by the tumors should induce a potent and diverse antitumor immunity able to prevent the immune escape mechanism of tumors. Finally, the vaccine should induce a long lasting memory response against the potential recurrence of the tumor.

EFIT predominantly utilizes activated CTLs since their normal effector function is to remove cells containing a pathological antigen which in this case is the tumor antigen. However, the induction of a CTL response is a highly regulated event and requires the concomitant involvement of both DCs and $CD4^+$ T helper cells. The DC is by far the most potent antigen presenting cell and activator of a CTL response[2] and the success or failure of a vaccine will partly depend on its proficiency at delivering sufficient quantities of peptide to them. For the most efficient presentation of the class I restricted peptides to the $CD8^+$ cells, the DCs ultimately must be activated. The in vivo activation of DCs normally occurs through the interaction of $CD4^+$ T helper cells with peptide bound MHC class II molecules and the reciprocal stimulation of the DCs via CD40-CD40L binding. The need for $CD4^+$ T helper responses can be circumvented through the use of other surrogate inducers of DC maturation including lipopolysaccharide(LPS), GM-CSF, or TNFα.[3,4] In either case, the success of EFIT depends on the ability of the vaccine to produce activated DCs loaded with MHC class I restricted peptides.

There are many advantages associated with the epitope focused approach to tumor immunotherapy in comparison to the use of vaccination with the entire antigen. Ishioka et al[5] found that vaccination with a minigene construct consisting of two HBV polymerase peptides induced a more potent CTL response than vaccination with the complete gene. The increased potency of an individual CTL should correlate with a greater anti-tumor efficacy since its cytolytic activity follows the law of mass action.[6] In other words, increases in the affinity of a CTL's TCR for a given peptide-MHC complex decreases the number of peptide-MHC complexes needed on the cell surface for T cell mediated lysis. Since the tumor will probably have a low expression of the peptides targeted by the vaccine, the increased potency will allow for the recognition of these cells by the induced CTLs whereas they would be cryptic to CTLs of lower affinity.

In addition to the increased strength of the immunity, different vaccination strategies involving epitope focusing induce a co-dominant induction of CTLs. The induction of a CTL response using a whole protein or tumor cell immunization predominantly produces an immunity centered around one immunodominant epitope.[7] In contrast, vaccination with a peptide vaccine based on dendritic cells,[8] DNA[5] or recombinant viruses [9,10] has shown to produce strong CTL induction to all included epitopes. This diverse immunity is important in disallowing the tumor an escape mechanism. Since the immunodominant epitope would be contained within one tumor antigen and restricted by one MHC haplotype, an immunity focused solely on that peptide could fail if the tumor mutates that epitope,[11] loses that tumor antigen[12,13] or downregulates that MHC molecule.[14,15] Using a vaccination protocol that elicits a potent CTL response against a broader profile of epitopes from the same or different antigens and different MHC haplotypes found on the tumor cells is unlikely to permit the tumor cells to evade the anti-tumor immunity.

Another advantage to EFIT is that the injected "beads on a string" constructs represent only portions of each tumor antigen that as a whole should not retain their biological activity. This is especially important in DNA based vaccines when the targeted antigen is an activated oncogene or a viral protein necessary for transformation. Even the use of non-tumorigenic proteins can not be considered completely safe since the effect of overexpressing certain tumor antigens is unknown. If an epitope is found to be problematic, it can simply be removed from the vaccine to eliminate its negative influences.

One caveat to the potentially safer use of epitope based vaccines concerns the use of an immunization strategy containing recombinant DNA constructs as a component of the vaccine. Although the expressed minigenes should not contain any ability to transform cells themselves, the injected DNA sequences can still be problematic. To achieve a high level of peptide expression, most DNA vectors employ a constitutively active viral promoter for transcription in all cell types. Should the DNA integrate into the host genome and bring the active promoter adjacent to a proto-oncogene, it could increase its expression and cause tumorigenesis. In a similar way, the disruption of the regulatory regions of certain proto-oncogenes via the integration of the DNA construct within them could have a similar effect.

The role epitope flanking sequences have on the presentation of distinct peptides remains controversial and can be considered both an advantage and a disadvantage. To extract only the minimal epitope from its surrounding sequences could remove it from residues inhibitory or necessary for presentation. Several groups have shown that alterations in the flanking amino acids change the presentation capabilities of the peptide.[16-19] Specifically, the juxtaposition of glycine or proline[19] residues has been found to be detrimental whereas neighboring alanines improve presentation.[17] In contrast to these studies, others have found no difference in the ability of certain peptides to be presented when they have been displaced to other proteins. Although the lack of complete knowledge in this area prevents definitive conclusions, it seems plausible that certain residues do affect the presentation of peptides. The reconciliation of the contrasting reports may be due to the fortuitous placement of the peptides between presentation facillatory residues in the studies where no effect of flanking sequences were seen.[20,21] The omission of the enhancing residues around the minimal epitope would be a disadvantage of EFIT except that many researchers have successfully used it in their vaccines. The discovery of residues with positive and negative influences on peptide presentation can be considered an advantage if this knowledge is used to design enhancing flanking sequences for each epitope in the "beads on a string" construct.

The fact that the peptides used in EFIT vaccines are haplotype restricted and that in the human population there exists a great diversity of MHC alleles indicates that a great amount of research must be performed in order to identify the necessary peptides. The broad spectrum of MHC alleles that a person expresses also complicates matters since each vaccine would need to be tailored to accommodate each patient's haplotype. These two obstacles have limited the application of an EFIT to people with one of the well studied MHC haplotypes which has hampered research in this area.

Peptide Vaccination

Peptide vaccination employs the injection of the minimal T cell epitopes as free peptides admixed with an adjuvant. This method began when Towsend et al[22] discovered that peptides bound to the MHC complex of cells was the recognizable element for CTLs. Simultaneously, Schulz et al[23] and Kast et al[24] independently proved the efficacy of these methods by showing that peptide vaccination could protect mice from challenge with an otherwise lethal dose of virus.

The method relies on the successful utilization of an adjuvant to create a depot after injection for the slow release of peptide in vivo. The peptides that comprise a vaccine include both the desired class I restricted peptides for the induction of the CTLs and class II restricted peptides for the induction of a helper T cell response. For proper induction of a CTL response, Keene et al[25] showed that a helper response was necessary. After the peptides leave the adjuvant depot, they passively move into the interstitial fluid and eventually bind to the MHC molecules found on DCs either in the vicinity or recruited to the area by some non-peptide aspect of the adjuvant itself. Once on the DCs, the class II peptides will induce a $CD4^+$ T helper cell

response that will reciprocally activate the DC. Now the activated DC with bound class I peptides will induce the CTL mediated immunity.

In 1993, Feltkamp et al[26] first used free peptide in adjuvant to induce a protective immunity against tumor cells in mice. After an exhaustive search involving the production and testing of every possible nonamer of the E6 and E7 oncoproteins of the human papilloma virus type 16, one peptide corresponding to amino acids 49-57 (RAHYNIVTF) of the E7 protein displayed high affinity for the H-2D^b MHC molecule. This peptide when emulsified in incomplete Freund's adjuvant and injected subcutaneously in mice provided protection against a subsequent challenge with C3, a cell line immortalized with the complete HPV 16 genome and activated EJ-ras. This peptide has proven efficacious in our laboratory in protecting against another cell line of mouse lung fibroblasts transfected with both the E7 and E6 proteins. However, despite the best efforts of many researchers, this peptide when used in a free peptide immunization has failed to show any therapeutic benefit in treating existing tumors.

In hopes of improving the potency of free peptide vaccinations, many alterations of the original immunization protocols have been tested. It could be argued that the passive diffusion of the peptides from the depot to the DCs may be a limiting factor in the vaccination's efficacy. With the recent discovery of class I restricted peptides bound to heat shock proteins[27] and the subsequent induction of tumor specific immunity through the vaccination of in vitro peptide pulsed HSPs, HSPs could serve as a chaperone to directly target the peptides to professional APCs.[28] Since the isolation of HSPs from tumor cells has already been shown to both protect[29] and eradicate existing tumors,[30] further manipulations to optimize the vaccination could have great beneficial effects for EFIT. Therapy has also been shown in a similar situation whereby peptide was complexed to a detoxified cellular invasive Bordatella pertussis adenylate cyclase, CyaA.[31] Theoretically, these methods overcome the therapeutic deficiencies of free peptide vaccination through the direct targeting of peptide to the class I pathway.

In addition to targeting the peptide to the professional APC, it has been found to be beneficial to increase the affinity of the peptides for the MHC molecule without altering its ability to stimulate a CTL response against the normal peptide. Alteration of the anchor residues of weak binding peptides towards the more conserved amino acids for a given haplotype increases the affinity[32-34] and does not affect the immunogenicity due to the deep and hidden nature of their binding.[35-38] Indeed three groups have shown that such a modification did not prevent the peptides from eliciting a CTL response to the normal peptide. Vierboom et al[39] showed that alteration of the anchor residues had no effect on the immunogenicity of the HPV 16 E7 $_{49-57}$ peptide. Valmori et al[40] showed that the increased HLA affinity resulted in a greater potency of CTL induction in cultures of peripheral blood lymphocytes immunized in vitro. Men et al[41] continued the analysis by showing that some of the peptide analogues induced greater CTL responses in HLA transgenic mice and that the induced CTL were able to cross react with cells pulsed with the normal peptide. In contrast, Clay et al[42] found that although the peptide analogues induced a greater CTL response, they were unable to cross react with the tumor cells. These results indicate that the use of peptide analogues in the future may be beneficial through their increased immunogenicity due to greater MHC affinity but only if the resultant CTLs recognize and lyse the tumor cells.

Another advance in the peptide vaccination protocol involves the use of different tactics to create a better environment for the induction of a CTL response. Due to the necessity for CD4$^+$ T helper cell mediated activation of dendritic cells to achieve optimal CTL priming and the decreased probability that the free class I and class II restricted peptides bind to the same DC, the covalent linkage of the two peptides was attempted. Shirai et al[43] showed in 1994, that the physical linkage of the two peptides produced greater CTL activity than vaccination with both peptides as single entities. Hypothetically, the linkage allows the presentation of both

peptides on the same DC allowing its subsequent activation and production of the appropriate Th1 cytokines for efficient CTL induction.

Production of the appropriate cytokine milieu through the inclusion of certain immunostimulatory sequences of DNA to the vaccine's adjuvant has also been shown to augment the induction of the CTLs. Specific DNA sequences found in prokaryotes containing unmethylated cytosine-guanine oligodinucleotides (CpG ODN) seem to act as a so called "danger signal" that alerts the immune system to the presence of an intracellular pathogen. The CpG ODN induce macrophages and DCs to produce IL-12, IL-18 and IFNα[44] of which the IL-12 has been shown to cause IFNγ secretion by natural killer cells.[45] The production of these Th1 type cytokines aids in the induction of a stronger CTL response when added to the adjuvant for peptide immunization.[46]

In addition to the success peptide vaccination has had in the induction of an antitumor immunity, there are many advantages to its usage. Peptides and their subsequent adjuvants tend to be relatively cheap and easy to produce. Since they are mostly injected as a subcutaneous dose, the actual mechanics of the vaccination are also rather simple and minimally invasive. The injected peptide and adjuvant also have been rather well tolerated in the ongoing clinical trials.[47] However, the greater success of the other methods of vaccination presented in this Chapter will probably shift the focus of strategies away from free peptide injections.

One disadvantage to the use of peptide vaccination as a method involves the observations of detrimental immune responses post vaccination. Vaccination of mice with a peptide corresponding to the LCMV is protective if given subcutaneously but tolerizing if given repeatedly i.p in high doses.[48] Although arguments as to the route of vaccination and concentration of cumulative peptide may confound these results, two papers by Toes et al[49,50] concerning tolerance induction by the E1A and E1B peptides of adenoviruses and one by Nieland et al[51] concerning the P1A peptide of P815 tumor cells are undeniable. Vaccination of mice with a peptide s.c. in IFA tolerized the tumor reactive T cells to the point where a normally regressor tumor grew out and killed the mouse whereas control animals survived unaffected after the tumor's characteristic regression. This disturbing observation was proven to be related to the pharmacokinetic behavior of these peptides that allowed them to rapidly spread throughout the body (Weijzen S and Kast WM unpublished observations). The induction of tolerance was also found to be method dependent as vaccination with the same peptides pulsed on DCs[52] or incorporated in a virus like particle (VLP)[51] prevented any outgrowth of tumor. This data reveals an inherent danger to the use of free peptide vaccination that is reversible when using other vaccination methods.

Another disadvantage to the use of peptide vaccination concerns the profile of induced CTL responses when a mixture of peptides are combined in one vaccine. As argued above, the most efficacious immunity should be diverse with strong responses to all epitopes to avoid tumor escape mechanisms through antigen loss, epitope mutation or HLA downregulation. Recent analysis of the immunodominance of five well characterized epitopes showed that injection of free peptides in IFA produced a hierarchy of induced CTL responses. The reversal of this immunodominant behavior between peptides was abrogated when other vaccination methods were employed.[8]

Other disadvantages to the use of peptide vaccination involve quality control issues regarding the actual composition of the vaccine. Gupta et al[53] discuss several difficulties with the use of free peptide emulsified in adjuvant. These problems derive from poor solubility of individual peptides, uncertainty in assuring a homogenous emulsification of peptide and problems of peptide formulation. In addition, to these issues, the relative paucity of approved and efficacious adjuvants for use in humans is also a limiting factor.

The sum extent of this discussion on peptide vaccination is that emulsification of peptides in adjuvant for subcutaneous injection was instrumental in progressing the field of tumor

immunotherapy to its current state but may not show great ability to treat preexisting cancers in clinical trials. The passive diffusion of the peptides from the depot to the DCs limits the effectiveness of the method for CTL induction. The increased capacity to induce CTLs with the use of synthetic peptide complexed to HSPs or Bordatella pertussis CyaA probably succeed due to their ability to target the peptides to the cytoplasm of the professional APCs. These two examples display the great importance of efficient loading of the peptides to DCs for therapeutic ability which is ultimately shown in the next section with the direct ex vivo loading of the peptides on isolated DCs.

Dendritic Cell Vaccination

Due to their critical role in the induction of a CTL response, dendritic cells are the logical choice for inclusion in an anti-cancer immunotherapeutic scheme. This approach was confounded by the difficulty in acquiring sufficient numbers of DCs until Inabu et al[54,55] reported on the in vitro production of large DC numbers from the coculturing of either peripheral blood mononuclear cells or bone marrow with high levels of GM-CSF. Improvements on the method that include either the addition of IL-4 to the GM-CSF or the in vivo stimulation of DCs via Flt3 ligand[56] have allowed for great increases in the biology of DC which has been directly applied to their use in EFIT approaches.[57]

Indifferent to the method used for the creation of the DCs, issues concerning their activation and antigen loading predict the eventual success or failure of the vaccine. Both Mackey et al[58] and Labeur et al[59] show that without the proper activation of DCs, they will lose their ability to induce an efficacious anti-tumor effect. Although DCs can be loaded using a variety of methods,[2] Morse et al[57] proved the critical dependence on the sequence of loading and activation depends on the nature of the immunizing antigen. Immunization with genetic material or coincubation of DCs with protein requires loading with subsequent activation whereas the exogenous loading of the DC's MHC molecules with peptides necessitate the opposite order of events. Activation of the DCs with a variety of methods including CD40 ligand, LPS or TNFα results in the upregulation of MHC class II, B7.1 and B7.2 molecules which were found to be necessary for CTL induction.[60] After the appropriate loading and activation of the DCs, they are infused into the patient where the activated DCs home towards secondary lymphoid organs. Herein they interact with the $CD8^+$ cells and cause the subsequent induction of peptide reactive CTLs.

The enormous potential of this method was best shown by Mayordomo et al[61] in 1995. In this landmark paper, synthetic peptide pulsed dendritic cells were able to protect against the injection of lethal doses of tumor cells in three distinct tumor models; MUT1 peptide for the Lewis lung carcinoma, 3LL, $E7_{49\text{-}57}$ for the HPV 16 genome transformed C3 tumor and $OVA_{257\text{-}264}$. In addition, the use of the peptide pulsed DCs were also able to eradicate existing day 7 3LL tumors and day 14 C3 tumors in mice. The sheer strength of the vaccine in the C3 model was shown when vaccination eliminated tumors in 60% and 20% of mice initially vaccinated 21 days and 28 days after tumor challenge. This paper provided the necessary proof to establish the use of peptide pulsed DCs as a forerunner in the quest to determine the ideal method for use as an immunotherapy in humans.

As a result, Murphy et al[62] recently reported the results of a phase II clinical trial where patients suffering from prostate cancer were treated with DCs pulsed with peptides derived from the prostate cancer marker, prostate specific membrane antigen (PSMA). Of the 33 subjects with stage D tumors who all no longer responded to current treatment regimens, 6 showed a partial response and 2 showed a complete response as based on the criteria dictated by the National Prostate Cancer Project. Although the fact that two partial responders and 1 com-

plete responder did not express the haplotype for which the peptides bound, HLA A*0201, confounds these results, the use of the vaccine shows great promise.

Exogenous loading of the MHC molecules with synthetic peptides is not the only loading method with proven efficacy. Using a p53 self peptide, Tuting et al[63] compared the use of exogenous peptide loading and DC transfection with a minigene DNA construct for their abilities to protect against a tumor challenge of a chemically induced p53-positive sarcoma. In their study, they report no difference in the efficacy of either method of loading in protecting the mice from tumor outgrowth.

The effectiveness of peptide loaded DCs vaccines in tumor immunotherapy may be due to one of the advantages of this strategy of immunization. Sandberg et al[8] showed that in contrast to the vaccination of mice with an admixture of five peptides emulsified in IFA, vaccination with DCs pulsed with the same admixture did not show any immunodominance. The exhibited co-dominant induction of CTL activity with peptide pulsed DCs versus peptide in IFA proved that immunodominance was neither a direct result of the biochemical makeup of the peptide nor its location with respect to other epitopes in the whole protein. Thus once the peptide reaches the surface of the DC bound in the MHC molecule, it has an equal chance of inducing a CTL response as every other binding peptide. This production of a diverse but equally potent CTL response may be one of the driving forces behind the great therapeutic ability of peptide pulsed DCs in EFIT.

The ex vivo manipulation is a disadvantage to this method of vaccination. The creation, loading and activation of the cells are time consuming expensive procedures that would require a large laboratory dedicated to the production of these vaccines. This is especially true if the vaccines prove efficacious in a number of cancers since they represent a major cause of illness in the United States. Thus the application of peptide loaded DCs although potentially effective cancer treatments, would involve a great undertaking to commonly apply.

A second disadvantage of DC based vaccination approaches involves the increased invasive nature of the creation and application of the vaccines. Since haplotype mismatched DCs would probably be less efficient vaccines than syngeneic cells, the source of the DCs would derive from the patient and involve either a blood draw or bone marrow tap. After the production and subsequent loading and activation, these DCs would probably be infused through an I.V. Although other vaccination routes may be employed, the relative success of the prostate cancer immunotherapy[62] and the finding that in mice the majority of the injected DCs given s.c. remained at the site of vaccination favor an intravenous application. The invasive nature of these vaccines would not preclude their usage if efficacious but could be less advantageous compared to an equally effective but noninvasive approach.

The use of a peptide loaded DC approach represents the most effective EFIT considering its ability to effectively treat mice with C3 tumors injected three weeks before initiation of therapy. Although the time and expense necessitated for their application are substantial, they represent only logistical impediments that could be overcome if the therapy is ultimately successful. The invasive nature of the method is also undesirable, but it is highly doubtful that a patient would refuse its employment if it meant the cure for their cancer. Thus, DC based immunotherapies currently represent the most effective EFIT to date.

DNA Vaccination

With the discovery by Wolff et al[64] of the potential to inject naked DNA into the muscles of mice for the stable expression of the inserted genes, the field of DNA vaccination was born. The efficacy of such an approach was illustrated by Ulmer et al[65] when they showed that injection of genes from the influenza virus protected mice from subsequent infections. This method was applied to tumor immunology with the demonstration of its protective ability to

prevent outgrowth of tumors containing either CEA[66] or MUC1[67] after vaccination with the respective genes. Consequently, DNA vaccination as a method of EFIT is now a heavily studied strategy.

Although the original route of vaccination involved injection of the genetic material i.m., Boyle et al[68] showed that a greater CTL response was induced earlier in intradermal vaccination versus the intramuscular route. Even though the induction of a CTL response via i.m. injection eventually reached the levels of the i.d. approach, it should be obvious that a faster induction of an immunity would be advantageous in terms of cancer treatment. The enhanced speed of induction via i.d. injection is most likely due to the great concentration of DCs in the skin versus the almost absent quantities in the muscle.[69]

The fate of the DNA after injection remains a controversy within the DNA vaccination field. It is an undeniable fact that the antigen produced by the injected DNA eventually reaches a DC for presentation to the T cells.[70,71] It is uncertain whether or not the injected DNA directly transfects DCs or is translated in surrounding tissues for the eventual uptake of the protein by the DCs in the draining lymph nodes.[72,73] In either case, the protein must reach the presentation pathways of the DCs.

The need for $CD4^+$ T helper cell stimulation is another unknown requirement for DNA vaccination. Although some groups report the direct need for $CD4^+$ help[5] others do not.[9] These discrepant results may be reconciled through the discovery of the inherent adjuvanticity of the DNA. It was finally shown that the presence of specific unmethylated cytosine-guanine oligodinucleotide containing sequences provided the immunostimulation necessary for proper CTL induction that is normally provided by $CD4^+$ T helper cells. The mechanism of action entails the production of IL-12 by the reacting DCs[44] that induces IFNγ release from NK cells.[45] Although the inclusion of MHC class II epitopes would not hurt the induction of an immunity via this method, the skewing of the cytokine milieu towards a Th1 pattern by either method allows the induction of peptide reactive CTLs. Once the DC is activated, it will extravasate from the tissues of the vaccination site and home towards the secondary lymphoid organs. Herein, the DCs activate the CTLs in an peptide dependent manner.

Thomson et al[9] was able to apply an epitope focused DNA vaccination towards a tumor immunotherapy. Using either an i.m. or i.d vaccination route, they were able to show that immunization with a DNA construct containing 8 class I restricted minimal epitopes induced co-dominant CTL induction for each peptide. The inclusion of the $_{264}$ epitope was able to induce a protective response against a lethal challenge of OVA transfected tumor cells. In addition, the inclusion of several viral peptides provided a OVA_{257} protective immunity against later challenge with vaccinia viruses containing the epitopes or the influenza virus.

Other groups also report on the successful use of minimal epitope containing DNA constructs in the induction of CTL responses proving that this is not an isolated phenomenon. Ciernek et al[74] used a minimal epitope containing DNA construct to show the induction of CTLs against a p53 peptide that induced tumor protection against P815 tumors transfected with the p53 peptide. In addition, Ishioka et al[5] recently reported on the successful use of a DNA construct to induce co-dominant CTL responses against HLA-A*0201 and HLA-A*1101 restricted peptides in human haplotype transgenic mice. This last report not only indicates the translation of the response to a quasi-human situation but also describes the use of an ER targeting signal to directly deliver the peptides to the class I MHC molecules.

To augment the induced immune response many different variations of the immunization protocol have been attempted. In another effort to target the peptides to the class I presentation pathway, Wu et al[75] found that the inclusion of an ubiquitin signal enhances the produced immunity theoretically through the increased turnover of the antigenic protein. Since the proper induction of a CTL response requires TH1 type cytokines, many groups have shown that the inclusion of genes for IL-2, IFNγ, IL-15 or GM-CSF have increased the magnitude of the

response.[76-78] Finally in an attempt to convert in vivo transfected cells not of the professional APC lineage to inducers of a CTL response, Corr et al[79] attempted to add the necessary costimulatory molecules through the coinjection of the B7.1 and B7.2 molecules with the minigenes. They found that inclusion of the B7.1 molecule gave the greatest potentiation of the CTL response to the peptide found in the minigene.

The potential for DNA vaccination as a method of EFIT is clearly strong. In addition to having measurable efficacy against tumors, the stable nature of the molecules also favors its future usage. With the commonplace use of recombinant DNA technology in today's scientific community, it is possible to easily manipulate the DNA constructs. Also, the defined isolation procedures for DNA make it a cost effective vaccine material since the cost of production is very small compared to other molecules.

In addition to the easy and cost effective attributes of DNA, the produced co-dominant induction of CTL responses to each peptide of a vaccine represents a major advantage. As detailed above in the introduction, the production of a diverse but potent response to all included peptides is key to the production of an efficacious vaccine. The ability of it to induce such a response indicates its potential for future application.

There are several disadvantages to the use of DNA as a vaccine strategy. In laboratories around the world, the integration of DNA into host cell genome is commonly performed through the simple introduction of the DNA into the cell. Since the method of DNA vaccination also relies on the introduction of foreign DNA into the host cells, the potential for integration is real. However, despite the intense effort of many researchers, no instance of integration mediated secondary malignancy induction following DNA vaccination has been reported. Although one strength of EFIT is the use of non-functional constructs to eliminate the introduction of oncogenic genes, the simple disruption of normal gene expression could occur through the interruption of its normal regulatory mechanisms. Although a rare occurrence, the integration into the genome could disregulate the expression of a proto-oncogene and initiate a new malignancy. Plus the use of viral promoters to drive high expression of the included multiepitope protein could insert close to an oncogene and increase its expression. These plausible situations represent one of the disadvantages to the use of DNA as a method of vaccination.

In some animal models, the injection of subimmunogenic amounts of DNA induced a state of unresponsiveness whereas in other models, the repeated injection of DNA lead to tolerance induction. These reports indicate that the use of DNA as a vaccine needs to be completely tested to insure that the concentration used will allow a normal immunizing response to occur. Other reports of DNA vaccination have shown that the injection of constructs in young animals have elicited a tolerizing effect that was not seen upon injection of the antigenic protein. This further illustrates the need to titrate the dose of DNA with the exact conditions for the immunization.

The antithesis to tolerance induction, autoimmunity, represents one last potential disadvantage to the use of DNA as a vaccine modality. It is possible that the injection and subsequent expression of the gene in muscle cells to elicit an immunity will cause the induced CTLs to destroy the transfected myocytes. Since the amount of muscle or skin transfected is not great, the induction of an autoimmune disease against the small percentage of cells would probably be well tolerated. Skin cells will regenerate from the stem cells found in the basal cell layer while the satellite cells of the muscle will grow and differentiate into new muscle fibers.

A more serious concern is the induction of anti-DNA antibodies via vaccination with DNA. Although mouse models exist where the injection of free DNA does increase the titers of anti-DNA antibodies, normal mice do not produce these antibodies unless the DNA is manipulated in certain artificial ways that would never be used in a DNA vaccination strategy. Thus although studies in animals indicate that the induction of an anti-DNA antibody

response and the eventual creation of a Lupus like syndrome is possible, it is not likely to occur with the vaccination of DNA for an immunizing response against the encoded antigen.

The above discussion of DNA as a method of EFIT reveals that it may be possible to elicit anti-cancer therapeutic immunities. Like the use of dendritic cells, the production of a co-dominant response is a major advantage that is partially responsible for its efficacy. The inherent immunostimulatory characteristic of DNA along with the easy addition of costimulatory help in the form of cytokines or secondary signals (i.e. B7.1, B7.2) make it an attractive option for future study. The disadvantages to its use, although potentially serious, may never become clinically relevant if the potential autoimmunity is delineated to small tissue regions around the vaccination site or left for theoretical discussions of anti-DNA antibodies.

Recombinant Virus Vaccination

Theoretically, the strong CTL response to viral proteins produced after infection with a virus should also be induced against proteins artificially introduced into the viruses genome. Indeed, mice were protected from a lethal challenge of LCMV after vaccination with recombinant vaccinia viruses engineered to express a polypeptide containing several LCMV epitopes.[80,81]

The recombinant viruses are engineered through the replacement of the normal genes necessary for viral replication with a genetic construct encoding the peptides of choice. Since these peptide expressing viruses are defective in terms of replication, they must be created in cultured cell lines that produce the viral structural genes replaced in the recombinant virus's genome by the polyepitope construct. These newly created, replication defective viruses are now injected into the host. Since the outer capsid is primarily responsible for the initial act of entering cells, these recombinant viruses still retain the ability to reach the cytoplasm. Once in the cytoplasm, the viral promoters are activated as usual and produce the polypeptide containing all of the peptides in great quantities. If the virus is normally tropic for DCs, the expression of the peptides in the cytoplasm facilitates entry to the class I pathway. For viruses that do not normally infect DCs, the overexpression of antigen can cause cell death and the release of cell debris or apoptotic bodies that are taken up by DCs and presented in a class I restricted manner.[82] In either case the requisite need for added $CD4^+$ T helper cell epitope of previous vaccination strategies may be unnecessary. Viral infections have the innate ability to activate DCs on their own[83-86] due to either strong endogenous helper epitopes or some unknown mechanism of DC activation. Regardless of the mechanism, both instances allow the expressed polyepitope protein to be processed by the dendritic cells for induction of a CTL response.

In an extension of the work by Oldstone[80] and Whitton,[81] Thomson et al[9] created a recombinant vaccinia virus containing epitopes from 5 different viruses, one parasite and the immunodominant peptide from ovalbumin (SIINFEKL). Infection of mice showed an equally potent CTL response to each peptide. Vaccination with this recombinant virus was shown to induce a protective tumor response against EL4 thymoma cells transfected with the ovalbumin protein but not parental EL4 cells. Use of this virus also produced a protective effect against viral challenges of murine cytomegalovirus (MCMV) and Sendai virus due to the inclusion of relevant epitopes from each virus in the epitope construct.

Due to health concerns of vaccinia usage discussed later, Toes et al[10] showed similar results using adenovirus and HPV derived peptides in a safer adenovirus vector. Inclusion of the dominant peptides $E1A_{234-243}$, $E1B_{192-200}$ and $E7_{49-57}$ in a recombinant adenovirus produced equally potent CTL responses to each peptide again displaying co-dominance. Vaccination with the recombinant vaccine was further shown to induce a protective immunity against a subsequent challenge with cell lines transformed with either Ad5E1A and activated EJ-ras or the HPV16 genome and activated EJ-ras. This result also showed that vaccination was almost equally potent if given s.c., i.m., or i.p. which alludes to the great potency afforded this vaccination method.

A new revolutionary strategy that involves the use of the outer virus coat to hypothetically retain the ability to enter the cytoplasm of infected cells like a normal virus employs virus-like particles (VLPs). Using the fact that the L1 and L2 proteins of the HPV virus spontaneously create empty capsids,[87,88] the covalent attachment of peptides to the inside moieties of the capsid proteins have proven efficacious. Peng et al[89] was able to show that bovine papillomavirus VLPs loaded with the $E7^{49-57}$ peptide could prevent the formation of EL4 thymoma tumor cells transfected with the E7 protein. Stronger evidence of the potency of the VLP method was shown by Nieland et al[51] whereby treatment of mice with HPV VLPs containing the P815 tumor peptide, P1A, showed therapy against day 5 and day 10 tumors. Other species viruses have also been used in successful vaccinations such as bovine papillomaviruses, canine oral papillomaviruses, cottontail rabbit papillomaviruses and Ty particles.[90] In addition to the introduction of peptides, Touza et al[91] have been able to load VLPs with DNA and show great ability to deliver the genetic material into cells. With these published reports, the prospect of using VLPs for future cancer immunotherapies is very strong indeed.

In addition to the great potency of the induced CTL response to infection with recombinant viruses expressing a multitude of peptides, there are several other advantages to this vaccination strategy. First, the infection of the host is a relatively simple procedure usually with minimal invasiveness. Although the best vaccination route may be to infect the patient in a similar manner to the normal infection route of the virus, Toes et al[10] showed that at least for adenoviruses, several different routes gave efficient vaccination. In addition, vaccines using adenoviruses have been tested extensively in army recruits[92,93] and found to be generally safe and non toxic.

Like the dendritic cell and DNA approaches, a positive feature of this method of vaccination is the ability to create a co-dominant induction of CTLs against the included epitopes. This diverse and equally potent response should be best suited to disallow the tumor any escape mechanisms. Thus, the use of recombinant viruses seems to be a potential method of future tumor immunotherapies.

There are, however, some disadvantages that need to be addressed before the method is applied. First, several poor outcomes have followed immunization with the vaccinia viruses. Vaccination was shown to produce encephalopathy and post vaccinal encephalitis in some individuals.[94] Its use also had the curious effect of decreasing the immunity of several younger individual's poxvirus immunity.[95] These negative side effects may be potentiated if the replication defective virus reverts to its wild type state. In a therapeutic setting, however, the benefit of eradication of the patient's cancer outweighs these potential risks.

Due to the necessity to produce these vaccines in packaging cells lines that contain the genes necessary for viral production, it is theoretically possible for recombination to create a reverted pathologic virus. Since it is impossible to check every individual virus produced, such an infectious virus may be injected into a human being. Although some of the concerns about reversion to wild type viruses may be alleviated through the use of relatively safer viruses (i.e. vaccinia, adenovirus), all viruses have the potential to cause problems. Even though it may be considered safer to use adenoviral vectors,[92,93] there are rare instances of acute hemorrhagic cystitis associated with adenoviral infections. Thus, perhaps only the use of the virus like particles that contain no genetic material may be considered safe.

A final disadvantage to the use of viral vectors for the induction of a CTL immunity concerns the issue of neutralizing antibody. For the virus to induce the CTL response, it must enter a viable cell to utilize its protein production machinery to make the polyepitope protein. Neutralizing antibody titers either in response to vaccination with the recombinant viruses or subsequent to previous infections with the wild type virus would prevent access to the cytoplasm of the cell. In data from both mouse[96] and human[97] studies, a preexisting immunity to vaccinia reduced the magnitude of the induced immune response after revaccination with

recombinant vaccinia virus vectors. This limiting effect of a preexisting immunity was overcome through an alteration in the route of immunization performed with the recombinant viral vaccine.[98] Thus in an oxymoronic manner, the undesirable humoral response to the viral vectors could inhibit the production of the desirable cellular immunity to the included epitopes.

The great immunity induced through the use of the viral vectors make their use in future immunotherapies highly probable. Although their potential negative side effects and the problem of neutralizing antibodies may initially limit their widespread usage, developing technology could make these disadvantages moot points. First, technologic advances may come up with a method of ensuring the exact production of the correct recombinant virus. To overcome the problem of neutralizing antibodies, the use of different strains of the same virus that do not share similar neutralizing antibody epitopes may be beneficial.[99,100] In addition, it has been possible to use similar viruses from different species to deliver the included epitopes without suffering the neutralizing antibody response of the previously used viral vector.[101] Thus, the use of recombinant viral vectors has shown great promise and most likely will eventually show good clinical effect in the immunotherapeutic treatment of cancer.

Conclusions

This Chapter dealt with a comparison of different vaccination strategies used in developing a successful epitope focused immunotherapy for cancer treatment. Since the efficacy of a vaccine will largely depend on the vaccine's ability to load the relevant peptides on the surface MHC class I molecules of activated DCs, each of the four sections attempted to discuss the route it would take to achieve that goal. In every case, the need for both the efficient loading of the peptides and the need for DC activation was discussed.

Free peptide in adjuvant showed success in protecting mice from tumor challenges but no therapy data. Associating free peptide with certain biologically active proteins like HSPs and Bordatella pertussis CyaA did show therapy against preexisting tumors. The latter results are most likely due to the ability of the peptide carriers to deliver the peptide to the DCs, a characteristic lacking in the injection of free synthetic peptide emulsified in adjuvant. It is no surprise then that the strongest data in support of EFIT as a potential clinical tool involved the direct ex vivo loading of synthetic peptides on DC for subsequent reinfusion. DNA vaccination using either the ex vivo transfection of DCs or the i.m or i.d. injection also proved to have great ability in creating potent CTL responses. Finally, the use of the recombinant viral vectors to deliver the "beads on a string" epitope constructs shows great promise most likely since CTL responses are the normal response to these intracellular pathogens.

Which of these methods will eventually become the method of choice for EFIT vaccination remains to be seen. It is interesting to note that the future vaccine may contain aspects of many of the different strategies to exploit the select advantages each method possesses. Schneider et al[102] showed that distinct strategies have unique potentials for satisfying the different requirements for CTL induction. Indeed in their study, they were able to show the increased efficacy of protecting mice against malaria through the initial vaccination with a naked DNA construct followed by a boost with a recombinant vaccinia virus containing the malarial antigens. Reversal of the order of vaccination actually revoked the CTL induction potential. This suggests that DNA vaccination may be better suited for initial priming of a CTL response and recombinant viral immunization for potentiating the T cells. Clear elucidation of the tasks best suited for each vaccination method may aid in the eventual production of a therapeutic vaccine.

It is an exciting time in EFIT with the great success certain investigators have had in treating mice with tumors and the publication of the initial reports from human clinical trials. Reports of absent clinical effect after certain vaccination trials are buoyed by the rationale that many trials are performed on end stage cancer patients whose cancers are refractory to current

therapeutic modalities. For example, in contrast to the nonexistent immunity induced in end stage cervical cancer patients immunized with a HPV 16 peptide based vaccine,[103] Muderspach et al[47] report better successes observed in women with CIN lesions using a similar vaccine. In hopes of applying a cancer vaccine earlier in the disease, our lab has shown that the vaccination of mice with a peptide vaccine will still produce an effective immunity given as soon as one day following the last radiation treatment (Small LA and Kast WM unpublished observations 1999). These results indicate that past failures of clinical trials of EFIT may be confounded by the accrual of end stage cancer patients and that future endeavors may benefit from the use of immunizations earlier in the disease progression.

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CHAPTER 5

Cancer Peptide Vaccines in Clinical Trials

Jeffrey S. Weber

Introduction

The revelation that protein antigens were processed into peptides by a pathway of intracellular degradation and presented on the surface of antigen presenting cells for recognition by T-cells in association with class I and II MHC molecules created a new paradigm for the generation and detection of antigen-specific immune responses in humans.[1-3] The subsequent discovery, cloning and identification of several classes of tumor-associated and tumor-specific antigens from human melanomas and other cancers has facilitated the performance of a number of clinical trials of peptide vaccines with and without adjuvants in patients with metastatic and resected melanoma and several other malignancies and pre-cancerous conditions. In this review I will summarize the different classes of melanoma and human papillomavirus (HPV) antigens that have been defined and describe the available data on recent attempts to boost immunity directed against defined melanoma and HPV antigens using peptides and detail their clinical significance. I will conclude with a proposal for an "optimal" vaccine schema and a call for an expansion of current peptide vaccine efforts in melanoma and other histologies.

Melanoma Antigens

The development of cancer vaccines has been most advanced in melanoma, which is unique among human tumors in the existence of compelling evidence for its immunogenicity. Spontaneous regression of primary melanomas is quite common, and the prognosis of cutaneous melanomas varies directly with the lymphocytic infiltrate.[4-5] Vitiligo is an autoimmune destruction of melanocytes that commonly occurs in melanoma patients, especially those who have been treated with interferon alpha or interleukin-2, and it is known to correlate with a favorable response to immunotherapy.[6-8] Tumor-reactive lymphocytes from patient peripheral blood or those which infiltrate metastatic melanoma lesions can be propagated in vitro as long term T cell lines or clones.[9-11] The ease with which such melanoma specific T-cells can be grown in vitro allowed Boon and colleagues in 1991 to describe the cloning of an antigen derived from a mutagenized melanoma cell line which was recognized by T cells.[12,13] This antigen was called MAGE, and was shown to define a family of antigens that had not previously been identified. MAGE-1 and several members of its multigene family were shown to be present on a significant proportion of melanoma cell lines and fresh tumors but were also found on a variety of tumors of epithelial and neuroectodermal origin as well as normal testis and placental tissue, but no other normal tissue.[14] MAGE, GAGE, BAGE and RAGE defined X-chromosome linked families of genes that were found respectively on melanoma, gastrointestinal, breast and renal cell tumors, many of which encoded antigens that were recognized by T-cells

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CHAPTER 12

Peptide Vaccines

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Introduction

For most of the history of vaccine production, the development of a new vaccine involved producing inactivated organisms or crude components of the pathogen. There is now generally a need to develop more precisely defined and novel vaccines against cancers and some pathogens (e.g., HIV), for which traditional approaches are unavailable or do not work well. With developments in recombinant DNA technology and cell biology, it is possible to dissect out the epitopes from the tumor cells and pathogens recognized by B- and T-cell receptors. These regions may be essential and effective for eliciting protective responses through neutralizing antibodies and T cell mediated immunity. This chapter will deal with the different peptide-based immunization strategies and their characteristics.

Molecular Basis for the Development of Peptide Vaccines

Epitopes recognized by B cells or neutralizing antibodies are usually classified as either continuous, consisting of a short linear fragment of an antigen, or discontinuous, comprising an assembly of amino acid residues brought together by folding of the protein chain. Most B-cell epitopes are discontinuous or conformational, which means that such epitopes require the full folded polypeptide for their presentation. In contrast, other epitopes are linear, being fully antigenic as short linear sequences in the range of 6- to 20-amino acid oligopeptides. A variety of techniques have been used to identify B-cell epitopes. The linear B-cell epitopes can be mapped with synthetic peptides, or direct sequencing of fragmented peptides recognized by the antibodies. Although discontinuous B-cell epitopes cannot be constructed within a short peptide, it is possible to identify small reactive peptides (mimotopes) that antigenically mimic the conformational immunogens by screening recombinant-based peptide libraries with antisera.[1-5] These linear epitopes and mimotopes are candidate vaccines for eliciting production of protective neutralizing antibodies by B cells.

While B cells recognize conformational or sequential epitopes on the surface of native proteins, T cells recognize only peptides derived from the processing of antigens in association with major histocompatibility complex (MHC) molecules.[6] However, there are major differences between the recognition of antigen by CD 4^+ and $CD8^+$ T cells, both in terms of the cellular site where the peptides are generated and the nature of MHC molecules required for their recognition.

$CD4^+$ T cells recognize peptides bound to MHC class II molecules on the surface of cells. Proteins derived from pathogens residing in intracellular vesicles are degraded by vesicular proteases into peptide fragments that bind to MHC II molecules for delivery to cell surface.[7,8] $CD4^+$ T cells also recognize peptide fragments derived from exogenous pathogens and proteins

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that are internalized into similar intracellular vesicles.[9] It has been recently reported that exogenous proteins can be degraded into antigenic peptides extracellularly by proteases secreted by dentritic cells (DCs) and loaded onto empty or peptide-receptive class II MHC molecules on the surface of DCs.[10]

$CD8^+$ T cells recognize peptides associated with MHC class I molecules. These peptides are derived from cytosolic proteins,[11] which are cleaved by proteasomes and translocated to the endoplasmic reticulum (ER) by peptide transporters associated with antigen processing (TAP) before final association with MHC class I molecules. The peptide-MHC class I complexes are then transported to the cell membrane where they can be recognized by $CD8^+$ T cells.[12] Certain professional antigen presenting cells (APCs) can also take up and present exogenous antigens through the MHC class I pathway to $CD8^+$ cells.[13,14]

On the basis of this knowledge of peptide processing and presentation, vaccines containing peptide epitopes recognized by $CD4^+$ and $CD8^+$ T cells have been developed.

Advantages and Disadvantages of Peptide-Based Vaccines

The peptide-based vaccine has a number of advantages, which include: Peptides can be chemically defined products and are relatively stable. They are relatively easy to manufacture and store. No infectious agent is involved in its manufacture. Any potential oncogenic or deleterious biological activity associated with whole pathogens or recombinant vaccines is avoided. Different molecules can be linked with peptides to enhance their immunogenicity.

The limitations of the peptide vaccines are: Many B cell epitopes are discontinuous, and adjacent molecules contribute to the epitopes. The conformation of a B-cell epitope in a protein may differ markedly from its shape as a free peptide. For a T-cell vaccine, this agent will need to contain multiple epitopes to cover the HLA diversity of target population, and to generate immunity for different epitope variants.

Adjuvants and Delivery Systems

Adjuvants have been used to increase the peptide-induced immune responses to the corresponding antigens. Although a number of adjuvants have been evaluated, only few, including incomplete Freund's adjuvant (IFA) and Montanide ISA, have been used in peptide-based clinical trials. Recently, molecularly defined agents have been shown to be promising adjuvants for peptide vaccines. Mouse models were used to evaluate large panels of molecularly defined adjuvants. These studies revealed that cytokines, IL-2 and IL-12,[15-17] are very potent in the ability to increase the efficacy of vaccines. GM-CSF also plays an important role in the induction and magnitude of cellular immune responses.[18] But in clinical trials, only peptide plus IL-2 was associated with an increase in clinical efficacy.[19,20] No enhancement in clinical efficacy was observed using GM-CSF or IL-12.[20]

Other non-cytokine immunomodulators involved in costimulation of T cells could be considered as candidate adjuvants. One of these, CD40 and its ligand (CD40L), has been shown to be important in DCs and B-cell activation, production of type 1 cytokines by T-helper cells, and generation of cytotoxic memory responses. The addition of CD40L to DNA vaccination was found to increase the antitumor efficacy.[21] The FLTs (tyrosine kinase receptor family) ligand can induce the apparent growth and differentiation of functional dendritic cells and has been reported to have antitumor effects.[22,23]

The B7-1 and B7-2 molecules expressed on the APCs play a critical role in controlling the activation or anergy of T cells. The engagement of B7 ligand, CD28 is associated with proliferation and differentiation, whereas an encounter with another B7 ligand, cytotoxic T-lymphocyte antigen-4 (CTLA-4) may trigger functional unresponsiveness.[24] In a recent report, soluble B7-IgG fusion proteins were shown to be effective in therapy of established tumors and

as a vaccine adjuvant in four mouse tumor models.[25] The blockade of the engagement of CTLA-4 has also been reported to potentiate immune responses to tumor cells.[26]

The delivery system that might lead to prolonged or pulsatile release of the peptides will reduce the frequency of immunization, and elicit comparable or greater immune responses. Keyhole limpet hemocyanin (KLH), which has been shown to recruit T helper cells and promote a memory cytotoxic T cell (CTL) response, has been used as a carrier in clinical trials. Liposomes that enable the introduction of lipid-soluble molecules or peptides to the immune system have been shown to induce both humoral and cell-mediated immune responses to a wide spectrum of antigens.[27] Poly-L-lactide co-glycolide (PLGA) is another suitable carrier to deliver peptides.[28] Immunostimulating complexes (ISCOMs) elicit humoral and cellular immunity, as well as CTL responses. Peptides incorporated into preformed ISCOMs-containing influenza virus-derived protein could stimulate specific immune responses.[29,30] Intact viruses, virus like particles (VLP), and recombinant plasmids also serve as carriers, which will be discussed in a later part of this chapter.

Design of Peptide Vaccines: Synthetic Peptides as B-Cell Vaccines

Initially, the development of peptides as possible vaccines was entirely directed to the production of neutralizing antibodies by the production of an epitope that would be recognized by B cells.[31,32] The concept was to identify and synthesize the epitope sequences of pathogen proteins that could form the candidate vaccines. In many cases, it has been possible to identify B-cell epitopes against which neutralizing antibodies are directed. The techniques of recombinant DNA combined with serological studies have enabled some epitopes to be mapped to precise amino acid residues. Linear B-cell epitopes of this type have been defined for the malarial circumsporozoite protein[33] and HIV-1 gp120.[34] Both of these polypeptides contain linear epitopes that are recognized by antibodies that neutralize the respective pathogens. However, some linear epitopes are only weakly immunogenic when presented in the context of full polypeptides. Such peptides would still be effective antigens if they were rendered more immunogenic.

The peptide can be conjugated to a carrier protein to increase its immunogenicity. The most commonly used carrier proteins in conjugates are bacterial proteins that humans commonly encounter, such as tetanus toxoid (TT), for which a conjugate with the malarial circumsporozoite epitope has been tested clinically.[35]

Increasing the number of the peptide groups in a conjugate can substantially increase immunogenicity particularly if the peptide epitopes are presented as a tandem array. A common approach is to form multiple antigenic peptides (MAP). A multimer of peptides from HIV gp120 having this structure was highly immunogenic.[36]

The immunogenicity of linear epitopes can also be increased by fusing the defined epitopes to a carrier protein that forms a large particle to improve the presentation of the peptide to cells of the immune system. The commonly used protein fusion partners of this type include HBsAg[37] and hepatitis B core antigen.[38]

The recombinant-based peptide libraries have been used to create the mimotopes for conformational B-cell epitopes that cannot be readily produced by recombinant or synthetic methods.[1-5] Peptide sequences were identified from the peptide library by the IgG antibodies (from patients with Graves' disease) that recognized the thyroid-stimulating-hormone receptor (TSHR).[39] These peptides were able to inhibit the cAMP synthesis that was induced by the IgGs from the same patient. Interestingly, these peptides do not resemble the linear sequence of TSHR and thus may mimic a spatial arrangement of the key antigenic residues. It has been recently reported that mimotopes can act as structural mimics of non-protein antigens and induce the production of anti-DNA antibodies.[40]

Peptide-Based T-Cell Vaccines. Identification of Peptide Epitopes Recognized by T Cells

The antigens recognized by T cells have been identified by the transfection of genomic DNA or cDNA library pools into cells expressing the appropriate MHC molecule and screened with antigen-specific T cells.[41,42] The candidate peptides from the antigens can be selected by computer programs[43,44] based on known MHC-binding motifs or synthesized as overlapping peptides if their MHC-binding motifs are unknown. Subsequently the peptides can be screened for their binding to MHC molecules.[45-47] The antigenic peptides are then determined by testing the ability of the binding peptides from the antigen to stimulate the cytokine secretion by the antigen-specific T cells. The drawback of this commonly used approach involves the requirement for determining the MHC restriction element for the antigen-specific T cells.

Antigenic peptides can be eluted with acid from either the antigen-expressing cell surface or purified peptide-MHC complexes, and subsequently separated by high-pressure liquid chromatography (HPLC). After pulsing onto APCs the eluted peptide fractions are tested for their ability to stimulate the antigen-specific T cells. The sequence of the peptide is then determined by Edman degradation or tandem mass spectrometry. A naturally processed epitope recognized by five melanoma-specific T-cell lines was identified by this method.[48] This approach has been limited by the need for highly specialized equipment and the requirement that peptides be present in sufficient quantity to enable their identification by these physical techniques.

Synthetic peptide libraries based on the MHC-binding motifs have been used for identification of antigenic peptides recognized by T cells.[49] However, the identified peptide may be a cross-reactive peptide, rather than a naturally processed peptide derived from an antigen.

Each of the three techniques mentioned above is dependent on the prior availability of T cells capable of recognizing the antigens, a requirement that often cannot be met. A new approach to the identification of antigenic peptides is involved in attempts to develop in vitro sensitization techniques.[50] Genes encoding candidate antigens can be transfected or transduced into APCs or synthesized peptides from candidate antigens based on known MHC-binding motifs can be pulsed onto APCs and used for in vitro sensitizations. If the generated T cells recognize the antigen-expressing cells, they can be used to identify the antigenic peptides. The peptides identified by this technique must have the ability to sensitize the T cells recognizing both the peptides themselves and the antigen-expressing cells.

A new possibility to identify T-cell epitopes is created by the use of HLA transgenic mice.[51] This approach involves screening the candidate peptides in the HLA transgenic mice, and subsequently testing these peptides in humans. Antigenic peptides have been identified by this technique and results indicate that the immunogenicity of peptides in transgenic mice resembles the immune responses against these peptides in humans bearing the same HLA haplotypes.[52-54] However, the peptides which are immunogenic in transgenic mice are not always immunogenic in humans.

Synthetic Peptides as T-Cell Vaccines

Synthetic peptides corresponding to epitopes recognized by T cells represent an ideal safe vaccine. Protective CTL responses induced by vaccination with MHC class I binding peptides was first reported by Schulz et al[55] for lymphocytic choriomeningitis virus (LCMV) and independently by Kast et al[56] for Sendai virus. In these studies, vaccination with peptides in IFA elicited substantial antiviral immune responses. Adjuvants were used to create a depot after injection for a slow release of peptides in vivo in these approaches. Much of the literature emphasizes the requirement for T-cell help for generating antipeptide immune reactivity. Conversely, some peptides appeared to induce CTL reactivity in the absence of obvious $CD4^+$ T cell help.[57] If a helper T-cell response is required to obtain an efficient CTL response, the

inclusion of general T-cell help agents (e.g., tetanus toxoid) or helper T cell epitopes (e.g., PADRE)[58,59] in peptide vaccines should be considered.

In hopes of improving the potency of free peptide vaccinations, many alterations of the original immunization protocols have been tested. It has been found that modification of certain residues of a peptide can enhance the immunogenicity of peptide through increasing its ability to bind MHC class I molecule without compromising the interaction of this complex with T-cell receptor.[60] Indeed, three groups have shown that such a modification enhanced the peptide's capacity for eliciting a CTL response.[61-63] In contrast, Clay et al[64] found that although the peptide analogues induced a greater CTL response, they were unable to cross react with the antigen-expressing cells. These results indicate that the use of modified peptide may only be beneficial if the resultant CTLs recognize not only the peptide analogue but also the cells expressing the authentic antigen.

Another advance in peptide vaccination involves the use of activated DCs to deliver the peptides. The DCs, expressing all costimulatory molecules necessary to efficiently initiate a cellular immune response, are by far the most potent antigen presenting cells.[65] DCs can be obtained by culturing either peripheral blood mononuclear cells or bone marrow cells with GM-CSF and IL-4. After the appropriate activation and loading of the DCs with peptides, they are infused back into the patient where the activated DCs home towards secondary lymphoid organs, interact with $CD8^+$ cells, and cause the subsequent induction of peptide reactive CTLs. The successful induction of protective immune responses by peptide loaded DCs have been shown in a number of studies.[66-70] In a recent clinical trial, 16 patients with metastatic melanoma were immunized with DCs pulsed either with immunodominant melanoma peptides or with melanoma lysates, and objective tumor regression was reported in five patients.[71]

Heat shock proteins (HSPs), which can bind the peptides and induce effective immune responses, have been used as chaperones to directly target the peptides to professional APCs.[72,73] CyaA, a detoxified cellular invasive Bordetella pertussis adenylate cyclase, has also been shown to have similar function.[74] These methods increase the efficacy of peptide vaccines probably through the direct targeting of peptides to the class I processing pathway. Other promising novel strategies may include the use of toxin-linked peptides,[75] T-helper sequence linked peptides,[76] and peptides attached to endoplasmic insertion signal sequences.[77]

It is important to realize that peptide vaccines do not always protect. Vaccination with certain peptides is associated with protective immunity in regimens[78-80] that can lead to tolerance in the case of other peptides[81-83] due to yet unknown mechanisms. When dealing with the peptides that are prone to induce tolerance, novel strategies (e.g., peptide-loaded DCs)[68] need to be considered.

Recombinant Vaccines Expressing T-Cell Epitopes

Minigenes encoding immunodominant peptide epitopes from the pathogens and tumor antigens can be engineered into recombinant virus and DNA. The injection of such recombinant constructs containing minigenes makes up a new way of epitope-based vaccination.

The recombinant viruses are engineered through the replacement of normal genes necessary for viral replication with minigenes encoding epitopes alone or in combination with endoplasmic reticulum insertion signals and immunomodulatory molecule sequences (such as cytokines and costimulatory molecules).[15,84-87] Thomson et al[88] created a recombinant vaccinia virus containing epitopes from different viruses, one parasite and the immunodominant peptide from ovalbumin. Vaccination with this recombinant virus showed potent CTL responses to each peptide, and induced protective responses against challenges of viruses and tumor cells expressing corresponding epitopes encoded by inserted minigenes. As all viruses have the potential to cause problems, the safety of the recombinant viruses should be consid-

ered and might be ensured in a number of ways. For example, some of the recombinant vaccines contain the viruses that are incapable of replicating in mammalian cells because of their host range[89] or removal of their viral genes critical for viral replication,[90] and some are highly attenuated viruses.[91] Another problem is that recombinant vaccines based on vaccinia and adenoviruses are likely to suffer from the pre-existing immunity. In data from both mouse[92] and human[93] studies, a preexisting immunity to vaccinia reduced the magnitude of the induced immune responses after revaccination with the recombinant vaccinia vectors. This limiting effect of a pre-existing immunity was overcome through an alteration in the route of immunization performed with the recombinant viral vaccine.[94] Another way of circumventing the problem is the use of viruses whose natural hosts are non-mammalian, such as the avian poxviruses.[95] It has also been possible to use similar viruses from different species to deliver the included peptides without suffering from the neutralizing antibody response of a previously used viral vector.(Kastetal,unpublished) space between Kast and et al

The T-cell epitopes can be delivered by recombinant plasmids containing the corresponding minigenes. Vaccination with recombinant plasmids coding for multiple T-cell epitopes have resulted in effective immune responses.[96,97] However, recombinant DNA is generally not as potent as recombinant viruses at eliciting effective immune responses. Important innovations concerning the design of these vectors include promoter optimization, enhancement of polyadenylation sequences, the removal of untranslated regions from the minigenes, and the use of intronic sequences to improve nuclear export. Other variations to augment the vaccine efficacy include the insertion of genes for IL-2, IL-12, IL-15, IFN-γ, and GM-CSF,[98-100] integration of DNA for B7.1 and B7.2,[101] inclusions of ubiquitin signals,[102] and incorporation of the "danger signal" sequence, unmethylated cytosine-guanine oligodinucleotides (CpG ODN).[103] Also DCs have been used to enhance the vaccination efficacy through directly transfecting DCs with epitope- containing plasmids.[104] Although no obvious adverse effects have been reported, these epitope-based DNA vaccines like all other DNA vaccines have the potential to induce anti-DNA autoimmune responses, and integrate their DNA to the host genomes.

Adoptive Cellular Therapy

The immunogenic peptides can be used to stimulate the peripheral blood lymphocytes in vitro to induce disease-specific CTLs. Once induced, the specific population can be expanded and then reinfused into the patient. This therapeutic approach may be beneficial to the patient who is immunosuppressed. This therapeutic approach is relatively costly since it must be individualized and because lymphocyte expansion is labor-intensive. Clinical trials in humans using CTLs that are specific for CMV have been conducted.[105,106] The CTLs utilized in these trials were induced using virus-infected fibroblasts as APCs. Early results showed that these treatments are safe and efficacious in preventing CMV infection. Clinical trials of adoptive immunotherapy have been reported for prevention and treatment of Epstein-Barr virus, HIV and human cancers.[107-111] In these studies, the expanded cell population tends to be heterogeneous, and the specific CTL population varies from treatment to treatment. This inconsistency in CTL generation may account for the relatively low success rate of previous adoptive T-cell therapy approach.

New strategies in the peptide-based adoptive immunotherapy of cancers and infective diseases are now being explored. Lymphocytes from cancer or chronic virus-infected patients are stimulated in vitro with APCs that have been optimally loaded with the antigenic peptides of choice. Among APCs, the DCs have been shown to be effective in presenting the selected peptides to the CTL precursors.[66,67,70] The addition of cytokines such as IL-7, and IL-12 at the early stage of the culture might facilitate the expansion of the CTL precursors. Several cycles of

antigen restimulation in the presence of IL-2 may be required to obtain the appropriate number of antigen-specific CTLs necessary for the adoptive transfer into the patients.

Another strategy is the use of modified peptides to sensitize CTLs for adoptive immunotherapy. The peptides, modified to increase their affinity for the MHC molecules, may have enhanced ability to stimulate the CTL precursor and preferably induce the CTLs with high avidity to antigens of pathogens and tumor cells. Indeed, the peripheral blood lymphocytes from melanoma patients stimulated with the modified gp100 peptide: g209-2M grow faster and have greater antitumor reactivity than T cells grown with the native g209 peptide.[60] A modified antigenic peptide derived from cyclophilin B was found to have increased ability in the induction of CTLs compared to the native peptides.[112]

The use of clonal population of CTLs represents another new strategy. From the bulk population of induced CTLs, individual clonal populations of CTLs can be derived, and assayed for their antigen activity. The T-cell clones with the highest apparent avidity can be expanded for adoptive transfer. This approach may have the advantage of generating a nearly uniform capacity to recognize the pathogens and tumor cells.

In addition to the use of a population of $CD8^+$ CTLs for adoptive transfer, $CD4^+$T cells can also be included in this therapy to enhance its efficacy. In studies using T cells specific for CMV, Waltel et al[113] have reported that the cytotoxic activity of adoptively transferred $CD8^+$ clones declined in patients deficient in helper $CD4^+$T cells specific for CMV. These results suggested the $CD4^+$T cell help is needed for the persistence of transferred $CD8^+$T cells. For this aim, epitopes from pathogens and tumor cells recognized by $CD4^+$T cells are required. Attempts to clone the molecular targets of $CD4^+$T cells have already met with considerable success.[114-118]

Summary and Perspectives

It has been shown in animal studies that peptide-based vaccines are capable of affording protection against infectious disease and cancer, as well as in control of these diseases once they have been established. The following challenge is to translate these results into prophylactic and therapeutic agents applicable to human diseases. We think that the use of selected antigenic peptides to elicit neutralizing antibodies and specific CTL responses will play an important role in vaccine development in certain fields.

For some infectious diseases and cancers, peptide-based preparations seem to offer the best hope for vaccination development. Some pathogens and tumor cells contain the epitopes recognized by neutralizing antibodies and T cells. But in many cases, it is very difficult to isolate the proteins containing the epitopes to use as immunogens for immunotherapy. In addition, peptides are relatively safe molecules. Administration of a short amino acid fragment derived from a pathogen or tumor cell offers fewer safety risks than the use of attenuated pathogens, full-length nucleic acids, or recombinant proteins, which are more likely to retain inherent biological activities.

The use of antigenic peptides also has a capacity to specifically manipulate the immune system. There are only a few potential epitopes within an antigen, and the capacity to deliver selected antigenic peptides at relatively high concentrations in immunogenic formulations is likely to be important in initiating and boosting an immune response where disease already exists. The peptide approach may also be useful for targeting immune responses to epitopes that are underrepresented or non-existent in the responses normally induced during infection or oncogenesis.

It is very important to test immunotherapy in combination with other therapeutic approaches in the management of human diseases. For instance, in the chronic viral disease settings, it may be important to use antigen-specific immunotherapy along with antiviral drugs

that are capable of reducing the viral replication. Similarly, combining forms of immunotherapy may be important in certain disease settings. For example, in cancer therapy, the patients may first be primed with the antigenic peptides using suitable formations, their lymphocytes will be expanded to tumor-specific CTLs in vitro, delivered back, and finally be boosted periodically with peptide to maintain high level of anti-tumor immune responses. Distinct strategies may have unique potentials for satisfying the different requirements for induction of protective immunity. In a recent study, Schneider et al[119] were able to show the increased efficacy of protecting mice against malaria through the initial vaccination with a naked DNA construct followed by a boost with a recombinant vaccinia virus containing the malaria antigens. Reversal of the order of vaccination actually revoked the CTL induction potential. This suggested that optimization of the combination and the method of vaccination may enhance protective immune responses.

For peptide vaccines, more studies are required to define the ideal combination of peptides and the best antigenic formulation, as well as a more appropriate selection of the patients and optimization of the immune monitoring. In addition, active vaccination should be tried as early as possible in patients with cancers and some infectious diseases. Indeed, most of the preclinical studies in animal models and the results of preliminary clinical trials have demonstrated that active immunotherapy has more chance of success when patients bear minimal tumor or infection burden.

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